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PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**UTILITY APPLICATION AND APPLICATION FEE TRANSMITTAL (1.53(b))**

COMMISSIONER FOR PATENTS

Box Patent Application  
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

Named Inventor(s) and  
Address(es):

James Anthony, Attila Lorincz, John Troy and Yanlin Tang

For:

DETECTION OF NUCLEIC ACIDS BY TYPE-SPECIFIC HYBRID CAPTURE  
METHOD

Enclosed are:

☒ 40 page(s) of specification, 1 page(s) of Abstract, 7 page(s) of claims

☐ 9 sheets of drawing ☐ formal ☒ informal

☐ 9 page(s) of Declaration and Power of Attorney

☐ Unsigned

☒ Newly Executed

☐ Copy from prior application

☐ Deletion of inventors including Signed Statement under 37 C.F.R. § 1.63(d)(2)

☐ Incorporation by Reference: The entire disclosure of the prior application, from which a copy of the combined declaration and power of attorney is supplied herein, is considered as being part of the disclosure of the accompanying application and is incorporated herein by reference.

☐ Microfiche Computer Program (Appendix)

☒ 54 page(s) of Sequence Listing

☒ computer readable disk containing Sequence Listing

☒ Statement under 37 C.F.R. § 1.821(f) that computer and paper copies of the Sequence Listing are the same

- ☐ Claim for Priority
- ☐ Certified copy of Priority Document(s)
- ☐ English translation documents
- ☐ Information Disclosure Statement
- ☐ Copy of \_\_\_\_ cited references
- ☐ Copy of PTO-1449 filed in parent application serial No. \_\_\_\_\_.
- ☐ Preliminary Amendment
- ☒ Return receipt postcard (MPEP 503)
- ☒ Assignment Papers (assignment cover sheet and assignment documents)
- ☒ A check in the amount of \$40.00 for recording the Assignment.
- ☐ Assignment papers filed in parent application Serial No. \_\_\_\_\_.
- ☐ Certification of chain of title pursuant to 37 C.F.R. § 3.73(b).
- ☐ This is a ☐ continuation ☐ divisional ☐ continuation-in-part (C-I-P) of prior application serial no. \_\_\_\_\_.
- ☐ Cancel in this application original claims \_\_\_\_\_ of the parent application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
- ☐ A preliminary Amendment is enclosed. (Claims added by this Amendment have been properly numbered consecutively beginning with the number following the highest numbered original claim in the prior application.
- ☐ The status of the parent application is as follows:
- ☐ A Petition For Extension of Time and a Fee therefor has been or is being filed in the parent application to extend the term for action in the parent application until \_\_\_\_\_.
- ☐ A copy of the Petition for Extension of Time in the co-pending parent application is attached.
- ☐ No Petition For Extension of Time and Fee therefor are necessary in the co-pending parent application.
- ☐ Please abandon the parent application at a time while the parent application is pending or at a time when the petition for extension of time in that application is granted and while this application is pending has been granted a filing date, so as to make this application co-pending.
- ☐ Transfer the drawing(s) from the patent application to this application.
- ☐ Amend the specification by inserting before the first line the sentence:  
This is a ☐ continuation ☐ divisional ☐ continuation-in-part of co-pending application Serial No. \_\_\_\_\_  
\_\_\_\_\_ filed \_\_\_\_\_.

## I. CALCULATION OF APPLICATION FEE (For Small Entity)

	Number Filed		Number Extra	Rate	Basic Fee
Total Claims	55	-20=	35	x\$9.00	\$345.00
Independent Claims	5	-3=	2	x\$39.00	\$78.00
Multiple Dependent Claims					
	[ X ] yes		Additional Fee =	\$130.00	\$130.000
	[ ] no		Add'l Fee =	NONE	

Total: \$ 868.00

- [ X ] A statement claiming small entity status is attached or has been filed in the above-identified parent application and its benefit under 37 C.F.R. § 1.28(a) is hereby claimed. Reduced fees under 37 C.F.R. § 1.9(F) (50% of total) paid herewith \$ 868.00.
- [ X ] A check in the amount of \$ 868.00 in payment of the application filing fees is attached.
- [ ] Charge Fee(s) to Deposit Account No. 13-4500. Order No. \_\_\_\_\_. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.
- [ X ] The Assistant Commissioner is hereby authorized to charge any additional fees which may be required for filing this application, or credit any overpayment to Deposit Account No. 13-4500, Order No. 2629-4017. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.

Respectfully submitted,

MORGAN &amp; FINNEGAN, L.L.P.

Dated: June 15, 2000By: Haiyan ChenHaiyan Chen  
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PATENT

Docket No. 2629-4017

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) or Patentee(s) : Anthony et al. Group Art Unit: To Be Assigned

Serial No. or Patent No. : To Be Assigned Examiner: To Be Assigned

Filed or Issued : June 15, 2000

For : DETECTION OF NUCLEIC ACIDS BY TYPE-SPECIFIC HYBRID CAPTURE METHOD

EXPRESS MAIL CERTIFICATE

Express Mail Label No. EJ606941808US

Date of Deposit: June 15, 2000

I hereby certify that the following attached paper(s) and/or fee

1. Specification (40 pgs); claims (7 pgs); abstract (1 pg); informal drawings (9 pgs);
2. Utility Application and Application Fee Transmittal (3 pages);
3. Paper copy of Sequence Listing ( 54 pages);
4. Computer readable disk containing Sequence Listing;
5. Statement Under 37 C.F.R. § 1.821(f) that computer and paper copies of Sequence Listing are the same
6. Combined Declaration and Power of Attorney (executed) (9 pages);
7. Statement Claiming Small Entity Status (2 pages);
8. Checks in the amount of \$868.00;
9. Recordation Form Cover Sheet (2 pages);
10. Assignment of Application for Patent (5 pages);
11. Check in the amount of \$40.00; and
12. Return Postcard

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to the Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Haiyan Chen

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mailing paper(s) and/or fee)

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PATENT

Docket No. 2629-4017

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) or Patentee(s) : James Anthony et al.

Group Art Unit: To Be Assigned

Serial No. or Patent No. : To Be Assigned

Examiner: To Be Assigned

Filed or Issued : June 15, 2000

For : DETECTION OF NUCLEIC ACIDS BY TYPE-SPECIFIC HYBRID CAPTURE METHOD

STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
37 CFR §1.97(f) AND §1.27 (c) – SMALL BUSINESS CONCERN

I hereby state that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below

NAME OF CONCERN Digene Corporation

ADDRESS OF CONCERN 1201 Clopper Road,

Gaithersburg, Maryland 20878

I hereby state that the above identified small business concern qualifies as a small business concern as defined in 13 CFR §§ 121.3-18, and reproduced in 37 CFR § 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both. I hereby state that exclusive rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled:

DETECTION OF NUCLEIC ACIDS BY TYPE-SPECIFIC HYBRID CAPTURE METHOD by

inventor(s) James Anthony, Attila Lorincz, John Troy and Yanlin Tang

described in

☒ the specification filed herewith

☐ application Serial No. \_\_\_\_\_, filed \_\_\_\_\_

☐ Patent No. \_\_\_\_\_, issued \_\_\_\_\_

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

NAME \_\_\_\_\_

ADDRESS \_\_\_\_\_  
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. 1.28(b))

NAME OF PERSON SIGNING Evan Jones

TITLE OF PERSON IF OTHER THAN OWNER President and CEO

ADDRESS OF PERSON SIGNING Digene Corporation  
1201 Clopper Road, Gaithersburg, Maryland 20878

SIGNATURE  DATE 6/12/00

FORM: SMALL.BUS  
 Rev. 05/26/98

\* NOTE: Separate statements are required from each name person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

## **DETECTION OF NUCLEIC ACIDS BY TARGET-SPECIFIC HYBRID CAPTURE METHOD**

### **FIELD OF INVENTION**

This invention relates to the field of nucleic acid detection methods in general and more particularly relates to the detection of nucleic acids by target-specific hybrid capture method.

### **BACKGROUND OF THE INVENTION**

The detection of specific nucleic acid sequences present in a biological sample is important for identifying and classifying microorganisms, diagnosing infectious diseases, detecting and characterizing genetic abnormalities, identifying genetic changes associated with cancer, studying genetic susceptibility to disease, and measuring response to various types of treatment. A common technique for detecting and quantitating specific nucleic acid sequences is nucleic acid hybridization.

Various hybridization methods are available for the detection and study of nucleic acids. In a traditional hybridization method, the nucleic acids to be identified are either in a solution or affixed to a solid carrier. The nucleic acids are detected using labelled nucleic acid probes which are capable of hybridizing to the nucleic acids. Recently, new hybridization methods have been developed to increase the sensitivity and specificity of detection. One example is the hybrid capture method described in U. S. Application Serial No. 07/792,585. Although these new hybridization methods offer significant improvements over the traditional methods, they still lack the ability to fully discriminate between highly homologous nucleic acid sequences.

It is therefore an object of the present invention to provide a hybridization method which is not only rapid and sensitive, but is also highly specific and capable of discriminating highly homologous nucleic acid target sequences.

### **SUMMARY OF THE INVENTION**

The present invention provides a novel nucleic acid detection method, referred to herein as target-specific hybrid capture ("TSHC"). TSHC is a highly

specific and sensitive method which is capable of discriminating and detecting highly homologous nucleic acid target sequences.

In one embodiment, the method relates to detecting a target nucleic acid wherein the targeted nucleic acid is hybridized simultaneously, or sequentially, to a capture sequence probe and an unlabelled signal sequence probe. These probes hybridize to non-overlapping regions of the target nucleic acid and not to each other so that double-stranded hybrids are formed. The hybrids are captured onto a solid phase and detected. In a preferred embodiment, an DNA-RNA hybrid is formed between the target nucleic acid and the signal sequence probe. Using this method, detection may be accomplished, for example, by binding a labeled antibody capable of recognizing an DNA-RNA hybrid to the double-stranded hybrid, thereby detecting the hybrid.

In another embodiment, the signal sequence probe used in the detection method is a nucleic acid molecule which comprises a DNA-RNA duplex and a single stranded nucleic acid sequence which is capable of hybridizing to the target nucleic acid. Detection may be accomplished, for example, by binding a labeled antibody capable of recognizing the DNA-RNA duplex portion of the signal sequence probe, thereby detecting the hybrid formed between the target nucleic acid, the capture sequence probe and the signal sequence probe.

In yet another embodiment, the signal sequence probe used in the detection method is a molecule which does not contain sequences that are capable of hybridizing to the target nucleic acid. Bridge probes comprising sequences that are capable of hybridizing to the target nucleic acid as well as sequences that are capable of hybridizing to the signal sequence probe are used. In this embodiment, the signal sequence probe comprises a DNA-RNA duplex portion and a single stranded DNA sequence portion containing sequences complementary to sequences within the bridge probe. The bridge probe, which hybridizes to both the target nucleic acid and the signal sequence probe, therefore serves as an intermediate for connecting the signal sequence probe to the target nucleic acid and the capture sequence probe hybridized to the target nucleic acid.

In another embodiment of the TSHC method of the invention, blocker probes comprising oligonucleotides complementary to the capture sequence probes are



used in the method to eliminate excess capture sequence probe, thereby reducing the background signal in detection and increasing specificity of the assay.

The present invention also relates to novel probes. These probes are nucleic acid sequences which can function in various hybridization assays, including, for example, the TSHC assay.

### **BRIEF DESCRIPTION OF THE DRAWING**

Figure 1 is a schematic diagram illustrating one embodiment of the target-specific hybrid capture method.

Figure 2 is a schematic diagram illustrating one embodiment of the target-specific hybrid capture method.

Figure 3 is a schematic diagram illustrating possible mechanisms of action of an embodiment that employs fused capture sequence probes in target-specific hybrid capture detection.

Figure 4 shows the analytical sensitivity and specificity of target-specific hybrid capture detection of HSV-1.

Figure 5 shows the analytical sensitivity and specificity of target-specific hybrid capture detection of HSV-2.

Figures 6A-6D show the various embodiments of the target-specific hybrid capture-plus method.

### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides a method for detecting the presence of nucleic acids in test samples. More specifically, the invention provides a highly specific and sensitive method which is capable of discriminating and detecting highly homologous nucleic acid sequences.

Any source of nucleic acid, in purified or non-purified form, can be utilized as the test sample. For example, the test sample may be a food or agricultural product, or a human or veterinary clinical specimen. Typically, the test sample is a biological fluid such as urine, blood, plasma, serum, sputum or the like. Alternatively the test sample may be a tissue specimen suspected of carrying a nucleic acid of

interest. The target nucleic acid in the test sample may be present initially as a discrete molecule so that the sequence to be detected constitutes the entire nucleic acid, or may only be a component of a larger molecule. It is not necessary that the nucleic acid sequence to be detected be present initially in a pure form. The test sample may contain a complex mixture of nucleic acids, of which the target nucleic acid may correspond to a gene of interest contained in total human genomic DNA or RNA or a portion of the nucleic acid sequence of a pathogenic organism which organism is a minor component of a clinical sample.

The target nucleic acid in a test sample can be DNA or RNA, such as messenger RNA, from any source, including bacteria, yeast, viruses, and the cells or tissues of higher organisms such as plants or animals. Methods for the extraction and/or purification of such nucleic acids are well known in the art. Target nucleic acids may be double-stranded or single-stranded. In the present method, it is preferred that the target nucleic acids are single-stranded or made single-stranded by conventional denaturation techniques prior to the hybridization steps of the method. In a preferred embodiment, base denaturation technique is used to denature the double-stranded target DNA.

The term "oligonucleotide" as the term is used herein refers to a nucleic acid molecule comprised of two or more deoxyribonucleotides or ribonucleotides. A desired oligonucleotide may be prepared by any suitable method, such as purification from a naturally occurring nucleic acid, by molecular biological means, or by de novo synthesis. Examples of oligonucleotides are nucleic acid probes described herein.

Nucleic acid probes are detectable nucleic acid sequences that hybridize to complementary RNA or DNA sequences in a test sample. Detection of the probe indicates the presence of a particular nucleic acid sequence in the test sample. In one embodiment, the target-specific hybrid capture method employs two types of nucleic acid probes: capture sequence probe (CSP) and signal sequence probe (SSP). A capture sequence probe comprises a nucleic acid sequence which is capable of hybridizing to unique region(s) within a target nucleic acid and being captured onto a solid phase. A signal sequence probe comprises a nucleic acid sequence which is capable of hybridizing to regions within a target nucleic acid that are adjacent to the

unique regions recognized by the CSP. The sequences of CSP and SSP are selected so that they would not hybridize to the same region of a target nucleic acid or to each other.

In addition, the CSP and the SSP are selected to hybridize to regions of the target within 50,000 bases of each other. The distance between the sequence to which the CSP hybridizes within the target nucleic acid and the sequence to which the SSP hybridizes is preferably between 1 to 50,000 bases, more preferably, the distance is less than 3,000 bases. Most preferably, the distance is less than 1,000 bases.

The CSP used in the detection method can be DNA, RNA, peptide nucleic acids (PNAs) or other nucleic acid analogues. PNAs are oligonucleotides in which the sugar-phosphate backbone is replaced with a polyamide or "pseudopeptide" backbone. In a preferred embodiment, the CSP is DNA. The CSP has a minimum length of 8 bases, preferably between 15 to 100 bases long, and more preferably between 20 to 40 bases long. The CSP is substantially complementary to the sequence within a target nucleic acid to which it hybridizes. The sequence of a CSP is preferably at least 75% complementary to the target hybridization region, more preferably, 100% complementary to this sequence. It is also preferred that the CSP contains less than or equal to 75% sequence identity, more preferably less than 50% sequence identity, to non-desired sequences believed to be present in a test sample. The sequence within a target nucleic acid to which a CSP binds is preferably 12 bases long, more preferably 20-40 bases long. It may also be preferred that the sequences to which the CSP hybridizes are unique sequences or group-specific sequences. Group-specific sequences are multiple related sequences that form discrete groups.

In one embodiment, the CSP used in the detection method may contain one or more modifications in the nucleic acid which allows specific capture of the probe onto a solid phase. For example, the CSP may be modified by tagging it with at least one ligand by methods well-known to those skilled in the art including, for example, nick-translation, chemical or photochemical incorporation. In addition, the CSP may be tagged at multiple positions with one or multiple types of labels. For example, the CSP may be tagged with biotin, which binds to streptavidin; or digoxigenin, which binds to anti-digoxigenin; or 2,4-dinitrophenol (DNP), which

binds to anti-DNP. Fluorogens can also be used to modify the probes. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycoerythrin, phycoerythrin, rhodamine, Texas Red or other proprietary fluorogens. The fluorogens are generally attached by chemical modification and bind to a fluorogen-specific antibody, such as anti-fluorescein. It will be understood by those skilled in the art that the CSP can also be tagged by incorporation of a modified base containing any chemical group recognizable by specific antibodies. Other tags and methods of tagging nucleotide sequences for capture onto a solid phase coated with substrate are well known to those skilled in the art. A review of nucleic acid labels can be found in the article by Landegren, *et al.*, "DNA Diagnostics-Molecular Techniques and Automation", Science, 242:229-237 (1988), which is incorporated herein by reference. In one preferred embodiment, the CSP is tagged with biotin on both the 5' and the 3' ends of the nucleotide sequence. In another embodiment, the CSP is not modified but is captured on a solid matrix by virtue of sequences contained in the CSP capable of hybridization to the matrix.

The SSP used in the detection method may be a DNA or RNA. In one particular embodiment of the invention, the SSP and target nucleic acid form a DNA-RNA hybrid. Therefore, in this embodiment, if the target nucleic acid is a DNA, then the preferred SSP is an RNA. Similarly, if the target nucleic acid is RNA, then the preferred SSP is a DNA. The SSP is generally at least 15 bases long. However, the SSP may be up to or greater than 1000 bases long. Longer SSPs are preferred. The SSP may comprise a single nucleic acid fragment, or multiple smaller nucleic acid fragments each of which is preferably between 15 to 100 bases in length.

In another embodiment, the SSP used in the detection method comprises a DNA-RNA duplex and a single stranded nucleic acid sequence capable of hybridizing to the target nucleic acid (Fig. 6A). The SSP may be prepared by first cloning a single stranded DNA sequence complementary to sequences within the target nucleic acid into a single-stranded DNA vector, then hybridizing RNA complementary to the DNA vector sequence to generate a DNA-RNA duplex. For example, if M13 is used as the DNA vector, M13 RNA is hybridized to the M13 DNA sequence in the vector to generate a DNA-RNA duplex. The resulting SSP contains a

DNA-RNA duplex portion as well as a single stranded portion capable of hybridizing to sequences within the target nucleic acid. The single stranded DNA should be at least 10 bases long, and may be up to or greater than 1000 bases long. Alternatively, the DNA-RNA duplex portion of the SSP may be formed during or after the reaction in which the single stranded portion of the SSP is hybridized to the target nucleic acid. The SSP can be linear, circular, or a combination of two or more forms. The DNA-RNA duplex portion of the SSP provides amplified signals for the detection of captured hybrids using anti-DNA-RNA antibodies as described herein.

In yet another embodiment, the SSP used in the detection method is a molecule which does not contain sequences that are capable of hybridizing to the target nucleic acid. In this embodiment, bridge probes comprising sequences capable of hybridizing to the target nucleic acid as well as sequences capable of hybridizing to the SSP are used. The bridge probes can be DNA, RNA, peptide nucleic acids (PNAs) or other nucleic acid analogues. In one embodiment (Fig. 6B), the SSP comprises a DNA-RNA duplex portion and a single stranded portion containing sequences complementary to sequences within the bridge probe. The bridge probe, which is capable of hybridizing to both the target nucleic acid and the SSP, therefore serves as an intermediate for connecting the SSP to the target nucleic acid and the CSP hybridized to the target nucleic acid. The SSP may be prepared as described above. In another embodiment (Fig. 6C), the SSP used in the detection method comprises multiple sets of repeat sequences as well as a single stranded RNA sequence capable of hybridizing to the bridge probe. A DNA oligonucleotide probe containing sequences complementary to the repeat sequences may be used to hybridize to the SSP to generate the RNA-DNA duplex needed for signal amplification. In yet another embodiment (Fig. 6D), the bridge probe contains a poly(A) tail in addition to sequences which are capable of hybridizing to the target nucleic acid. The SSP used in this example comprises poly(dT) DNA sequences. The bridge probe therefore is capable of hybridizing to the SSP via its poly(A) tail. A RNA probe comprising poly(A) sequences may be used to hybridize to the remaining poly(dT) DNA sequences within SSP to form a RNA-DNA duplex. The SSP comprising poly(dT)

sequences and the RNA probe comprising poly(A) sequences are preferably 100 to 5,000 bases long.

The SSP used in the detection method of the invention can be unmodified, or modified as with the CSP using methods described above and/or known in the art. In a preferred embodiment, the SSP is a covalently unmodified probe.

It is understood that multiple CSPs and/or SSPs can be employed in the detection method of the invention.

In another embodiment, an oligonucleotide probe comprising complementary sequences of two or more distinct regions of the target nucleic acid are fused together and used as the capture sequence probe in the method of the invention. Alternatively a single probe can be designed and produced which contains sequences complementary to single or multiple target nucleic acids. This type of probe is also referred to herein as a "fused" CSP. As shown in Example 5, the fused capture sequence probe works as effectively as the combination of two unfused CSPs when used at the same concentration.

The nucleic acid probes of the invention may be produced by any suitable method known in the art, including for example, by chemical synthesis, isolation from a naturally-occurring source, recombinant production and asymmetric PCR (McCabe, 1990 In: *PCR Protocols: A guide to methods and applications*. San Diego, CA., Academic Press, 76-83). It may be preferred to chemically synthesize the probes in one or more segments and subsequently link the segments. Several chemical synthesis methods are described by Narang et al. (1979 *Meth. Enzymol.* 68:90), Brown et al. (1979 *Meth. Enzymol.* 68:109) and Caruthers et al. (1985 *Meth. Enzymol.* 154:287), which are incorporated herein by reference. Alternatively, cloning methods may provide a convenient nucleic acid fragment which can be isolated for use as a promoter primer. A double-stranded DNA probe is first rendered single-stranded using, for example, conventional denaturation methods prior to hybridization to the target nucleic acids.

Hybridization is conducted under standard hybridization conditions well-known to those skilled in the art. Reaction conditions for hybridization of a

probe to a nucleic acid sequence vary from probe to probe, depending on factors such as probe length, the number of G and C nucleotides in the sequence, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words more stringent conditions. Chapter 11 of the well-known laboratory manual of Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, second edition, Cold Spring Harbor Laboratory Press, New York (1990) (which is incorporated by reference herein), describes hybridization conditions for oligonucleotide probes in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity. Hybridization is typically performed in a buffered aqueous solution, for which conditions such as temperature, salt concentration, and pH are selected to provide sufficient stringency such that the probes hybridize specifically to their respective target nucleic acid sequences but not any other sequence.

Generally, the efficiency of hybridization between probe and target improve under conditions where the amount of probe added is in molar excess to the template, preferably a 2 to  $10^6$  molar excess, more preferably  $10^3$  to  $10^6$  molar excess. The concentration of each CSP provided for efficient capture is at least 25 fmoles/ml (25 pM) in the final hybridization solution, preferably between 25 fmoles to  $10^4$  fmoles/ml (10 nM). The concentration of each SSP is at least 15 ng/ml in the final hybridization solution, preferably 150 ng/ml. Table A shows the conversion of SSP concentrations expressed in ng/ml to molar basis.

Table A

Conversion of SSP Concentration From ng/ml to fmoles/ml

SSP Concentration in ng/ml	SSP Concentration in fmoles/ml (pM)	
	SSP is a 3kb RNA	SSP is a 5kb RNA
15 ng/ml	15.1	9
150 ng/ml	151	90
600 ng/ml	606	364

Hybridization of the CSP and the SSP to the target nucleic acid may be performed simultaneously or sequentially and in either order. In one embodiment, hybridization of the CSP and hybridization of the SSP to the target nucleic acid are performed simultaneously. The hybrid formed is then captured onto a solid phase coated with a substrate to which ligand attached to the CSP binds with specificity. In another embodiment, hybridization of the SSP to the target nucleic acid is performed after the hybridization of the CSP to the target nucleic acid. In this case, the CSP may be immobilized on a solid phase before or after hybridization. In this embodiment, both the CSP and the target may be bound to the solid phase during the SSP hybridization reaction.

It will be understood by those skilled in the art that a solid phase or matrix includes, for example, polystyrene, polyethylene, polypropylene, polycarbonate or any solid plastic material in the shape of plates, slides, dishes, beads, particles, cups, strands, chips and strips. A solid phase also includes glass beads, glass test tubes and any other appropriate glass product. A functionalized solid phase such as plastic or glass that has been modified so that the surface contains carboxyl, amino, hydrazide, aldehyde groups, nucleic acid or nucleotide derivatives can also be used. Any solid phase such as plastic or glass microparticles, beads, strips, test tubes, slides, strands, chips or microtiter plates can be used.

In one preferred embodiment, the CSP is labelled with biotin, and streptavidin-coated or avidin-coated solid phase is employed to capture the hybrid.



More preferably, streptavidin-coated microtiter plates are used. These plates may be coated passively or covalently.

The captured hybrid may be detected by conventional means well-known in the art, such as with a labelled polyclonal or monoclonal antibody specific for the hybrid, an antibody specific for one or more ligands attached to the SSP, a labelled antibody, or a detectable modification on the SSP itself.

One preferred method detects the captured hybrid by using an anti-RNA-DNA antibody. In this embodiment, the anti-RNA-DNA antibody is preferably labelled with an enzyme, a fluorescent molecule or a biotin-avidin conjugate and is non-radioactive. The label can be detected directly or indirectly by conventional means known in the art such as a colorimeter, a luminometer, or a fluorescence detector. One preferred label is, for example, alkaline phosphatase. Other labels known to one skilled in the art can also be employed as a means of detecting the bound double-stranded hybrid.

Detection of captured hybrid is preferably achieved by binding the conjugated antibody to the hybrid during an incubation step. Surfaces are then washed to remove any excess conjugate. These techniques are known in the art. For example, manual washes may be performed using either an Eppendorf™ Repeat Pipettor with a 50 ml Combitip™ (Eppendorf, Hamburg, Germany), a Corning repeat syringe (Corning, Corning, NY), a simple pump regulated by a variostat, or by gravity flow from a reservoir with attached tubing. Commercially available tube washing systems available from Source Scientific Systems (Garden Grove, CA) can also be used.

Bound conjugate is subsequently detected by a method conventionally used in the art, for example, colorimetry or chemiluminescence as described at Coutlee, *et al.*, *J. Clin. Microbiol.* 27:1002-1007 (1989). Preferably, bound alkaline phosphatase conjugate is detected by chemiluminescence by adding a substrate which can be activated by alkaline phosphatase. Chemiluminescent substrates that are activated by alkaline phosphatase are well known in the art.

In another embodiment, the target specific hybrid capture method of the invention employs blocker probes in addition to the CSP and SSP. A blocker probe comprises sequences that are complementary to the sequences of the CSP. The

sequence of a blocker probe is preferably at least 75% complementary to the sequence of the CSP, more preferably, 100% complementary to the CSP. The addition of the blocker probes to the hybridization reaction mixture prevents non-hybridized CSP from hybridizing to cross-reactive nucleic acid sequences present in the target and therefore increases the specificity of the detection.

The blocker probe is generally at least 5 bases long, preferably 12 bases long. The concentration of the blocker probe in the hybridization reaction is preferably in excess to that of the CSP and SSP. Preferably, the blocker probe is present in a 2-fold molar excess, although, it may be present in an up to 10,000-fold molar excess. The blocker probes can be DNA, RNA, peptide nucleic acids (PNAs) or other nucleic acid analogues.

In one embodiment, blocker probes complementary to the full-length or near full-length of the CSP are used. Following the reaction in which the hybrid between CSP, SSP and the target nucleic acid is formed, one or more blocker probes may be added to the reaction and the hybridization is continued for a desired time. The hybridization products are then detected as described above.

In another embodiment, blocker probes complementary to only a portion of the CSP and are shorter than the CSP are used. These blocker probes have a lower melting temperature than that of the CSP. Preferably, the melting temperature of the blocker probe is 10 degrees lower than that of the CSP. In this case, the blocker probe is preferably added to the target nucleic acids simultaneously with the CSP and the SSP. Since the blocker probe has a lower melting temperature than the CSP, the initial temperature for hybridization is chosen such that the blocker probe does not interfere with the hybridization of the CSP to its target sequences. However, when the temperature of the hybridization mixtures is adjusted below the temperature used for target hybridization, the blocker probe hybridizes to the CSP and effectively blocks the CSP from hybridizing to cross-reactive nucleic acid sequences. For example, when the hybridization products are incubated at room temperature on a streptavidin-coated microtiter plate during hybrid capture, the blocker probes may be added.

The following examples illustrate use of the present amplification method and detection assay and kit. These examples are offered by way of

illustration, and are not intended to limit the scope of the invention in any manner. All references described herein are expressly incorporated *in toto* by reference.

### **Example 1**

#### **Target-Specific Hybrid Capture (TSHC) Assay Protocol**

Herpes Simplex Virus 1 (HSV-1) and Herpes Simplex Virus 2 (HSV-2) viral particles of known concentration (Advanced Biotechnologies, Inc., Columbia, MD) or clinical samples were diluted using either Negative Control Media (Digene Corp., Gaithersburg, MD) or Negative Cervical Specimens (Digene). Various dilutions were made and aliquoted into individual microfuge tubes. A half volume of the Denaturation Reagent 5100-0431 (Digene) was added. Test samples were incubated at 65°C for 45 minutes for denaturation of nucleic acids in the samples.

Following denaturation, a hybridization solution containing signal sequence probes (SSPs) (600 ng/ml each) and capture sequence probes (CSPs) (2.5 pmoles/ml each) was added to the sample, and incubated at 74°C for 1 hour. Blocker probes in a solution containing one volume of 4x Probe Diluent (Digene), one volume of Denaturation Reagent and two volumes of the Negative Control Media were then added to the hybridization mixture and incubated at 74°C for 15 minutes.

In a second series of experiments, following denaturation of nucleic acids, a hybridization mixture containing SSPs (600 ng/ml each), CSPs (2.5 pmoles/ml each), and blocker probes (250 pmoles/ml each) was added to the samples and incubated for one hour at 74°C.

Tubes containing reaction mixtures were cooled at room temperature for 5 minutes, and aliquots were taken from each tube and transferred to individual wells of a 96-well streptavidin capture plate (Digene). The plates were shaken at 1100 rpms for 1 hour at room temperature. The supernatants were then decanted and the plates were washed twice with SNM wash buffer (Digene) and inverted briefly to remove residual wash buffer. The alkaline-phosphatase anti-RNA/DNA antibody DR-1 (Digene) was then added to each well and incubated 30 minutes at room temperature. The wells were then subjected to multiple wash steps which include: 1) three washes with Sharp wash buffer (Digene) at room temperature; 2) incubation of

the plate with the Sharp wash buffer for 10 minutes at 60°C on a heat block; 3) two washes with the Sharp wash buffer at room temperature; and 4) one wash with the SNM wash buffer (Digene) at room temperature. Following removal of the residual liquid, luminescent substrate 5100-0350 (Digene) was added to each well and incubated for 15 minutes at room temperature. The individual wells were then read on a plate luminometer to obtain the relative light unit (RLU) signal.

Solutions containing Negative Control Media or known HSV Negative Cervical Specimens were used as negative controls for the test samples. The signal to noise ratio (S/N) was calculated as the ratio of the average RLU obtained from a test sample to the average RLU of the negative control. The signal to noise ratio was used as the basis for determining capture efficiency and the detection of target nucleic acids. A S/N value of 2 or greater was arbitrarily assigned as a positive signal while a S/N values less than 2 was considered negative. The coefficient of variation (CV) which is a determination of the variability of the experiment within one sample set was calculated by taking the standard deviation of the replicates, dividing them by the average and multiplying that value by 100 to give a percent value.

The capture sequence probes and the blocker probes used in experiments described in Examples 2-13 were synthesized using the method described by Cook et al. (1988 Nucl. Acid. Res., 16: 4077-95). Unless otherwise noted, the capture sequence probes used in the experiments described herein were labeled with biotins at their 5' and 3' ends.

The signal sequence probes used in experiments described in Examples 2-13 are RNA probes. These probes were prepared using the method described by Yisraeli et al. (1989, Methods in Enzymol., 180: 42-50).

**Example 2**

The following tables describe the various probes used in experiments described in Examples 3-13.

**Table 1****HSV-1 Clones from which HSV-1 Probes are derived**

Clone Name	Host Vector	Cloning Site(s)	Insert Size (bp)	Sequence Location within HSV-1
RH3	Dgx3	Hind III, Eco RI	5720	39850-45570
R10	Blue Script SK+	Eco RI	4072	64134-68206
RH5B	Blue Script SK+	Eco RV, Eco RI	4987	105108-110095
H19	Blue Script SK+	Hind III	4890	133467-138349

**Table 2****HSV-2 Clones from which HSV-2 Probes are derived**

Clone Name	Host Vector	Cloning Site(s)	Insert Size (bp)	Sequence Location in HSV-2
E4A	Blue Script SK+	Bam HI	3683	23230-26914
E4B	Blue Script SK+	Bam HI Eco RI	5600	26914-32267
I8	Blue Script SK+	Hind III	2844	41624-44474
EI8	Dgx3	Hind III, Eco RI	3715	44474-48189
4L	Blue Script KS+	Bam HI, Eco RI	4313	86199-90512

**Table 3**  
**Capture Sequence Probes for HSV-1**

Probe	Sequence	Size (bp)	Location within HSV-1
TS-1	(TTATTATTA)CGTTCATGTCGGCAAACAGCT CGT(TTATTATTA) [SEQ ID NO:1]	24	105040-105063
TS-2	(TTATTATTA)CGTCCTGGATGGCGATACGGC (TTATTATTA) [SEQ ID NO:2]	21	110316-110336
VH-3	CGTCCTGGATGGCGATACGGC [SEQ ID NO:3]	21	110316-110336
NC-1	CGTTCATGTCGGCAAACAGCTCGT [SEQ ID NO:4]	24	105040-105063
VH-4 (fusion of VH3, NC-1)	CGTTCATGTCGGCAAACAGCTCGT- CGTCCTGGATGGCGATACGGC [SEQ ID NO:5]	45	105040-105063; 110316-110336
HZ-1	GATGGGGTTATTTTTCCTAAGATGGGGC GGGTCC [SEQ ID NO:6]	34	133061-133094
VH-2	TACCCCGATCATCAGTTATCCTTAAGGT [SEQ ID NO:7]	28	138367-138394
FD-1	AAACCGTTCATGACCGGA [SEQ ID NO:8]	19	39281-39299
RA-2	ATCGCGTGTTCAGAGACAGGC [SEQ ID NO:9]	22	39156-39177
NC-2	CAACGCCCAAATAATA [SEQ ID NO:10]	17	46337-46353
FD-2	GTCCCCGAaCCGATCTAGCG (note small cap a is mutated base) [SEQ ID NO:11]	20	45483-45502
RA-4	CGAACCATAAACCATTCCCCAT [SEQ ID NO:12]	22	46361-46382
ON-3	CACGCCCGTGGTTCTGGAATTCGAC [SEQ ID NO:13]	25	64105-64129
HZ-2	(TTTATTA)GATGGGGTTATTTTTCCTAAGATG GGGCGGGTCC [SEQ ID NO:14]	34	133061-133094
ZD-1	GGTTATTTTTCCTAAG [SEQ ID NO:15]	16	133064-133079
ZD-2	(ATTATT)GGTTATTTTTCCTAAG(ATTATT) [SEQ ID NO:16]	16	133064-133079
F6R	ACGACGCCCTTGACTCCGATTCGTCATCGGAT GACTCCCT [SEQ ID NO:17]	40	87111-87150
BRH19	ATGCGCCAGTGTATCAATCAGCTGTTTCGGGT [SEQ ID NO:18]	32	133223-133254
F15R	CAAAACGTCCTGGAGACGGGTGAGTGTCGGC GAGGACG [SEQ ID NO:19]	38	141311-141348
VH-1	GTCCCCGACCCGATCTAGCG [SEQ ID NO: 20]	20	45483 - 45502
ON-4	GCAGACTGCGCCAGGAACGAGTA [SEQ ID NO: 21]	23	68404 - 68426
PZ-1	GTGCCCACGCCCGTGGTTCTGGAATTCGACAG CGA [SEQ ID NO: 22]	35	64105 - 64139
PZ-2	GCAGACTGCGCCAGGAACGAGTAGTTGGAGT ACTG [SEQ ID NO: 23]	35	68404 - 68438
FG-2	AAGAGGTCCATTGGGTGGGGTTGATACGGGA AAGAC [SEQ ID NO: 24]	36	105069 - 105104
FG-3	CGTAATGCGGCGGTGCAGACTCCCCTG [SEQ ID NO: 25]	27	110620 - 110646
FG-4	CCAAC TACCCCGATCATCAGTTATCCTT AAGGTCTCTTG [SEQ ID NO: 26]	39	138362 - 138400

Hsv1-LF15R (SH-3)	(AAAAAAAAA)CAAAACGTCCTGGAGACGGGT GAGTGTGCGCGAGGACG [SEQ ID NO: 27]	38	141311-141348
Hsv1-F15-2B (GZ-1)	CAAAACGTCCTGGAGACGGGTGAGTGTGCGC GAGGACG [SEQ ID NO: 28]	38	141311-141348
Hsv1-F15-3B (GZ-2)	CAAAACGTCC-bio-U-GGAGACGGGTGAG TG-bio-U-CGGCGAGGACG [SEQ ID NO: 29]	38	141311-141348

\* Sequences in parentheses are "tail" sequences not directed at HSV.

**Table 4**  
**Blocker Probes for HSV-1**

Probe	Sequence	Size (bp)	Capture Probe to which it hybridizes
EA-1	AGGAAAAATAACCCCATC [SEQ ID NO:30]	18	HZ-1
EA-2	GACCCGCCCCATCTT [SEQ ID NO:31]	15	HZ-1
ZD-3	GGACCCGCCCCATCTTAGGAAAAATAAC CCCATC [SEQ ID NO:32]	34	HZ-1
NG-7	AAAAATAACCCCA [SEQ ID NO:33]	13	HZ-1
NG-8	CGCCCCATCTT [SEQ ID NO:34]	11	HZ-1
NG-4	CCATCTTAGGAAAAA [SEQ ID NO:35]	15	HZ-1
GP-1	ATAACTGATGATCGG [SEQ ID NO:36]	15	VH-Z
EA-3	CCACCCAATGGACCTC [SEQ ID NO: 37]	16	FG-2
EA-4	GTCTTTCCCGTATCAACC [SEQ ID NO: 38]	18	FG-2
EB-7	CGCCGCATTACG [SEQ ID NO: 39]	12	FG-3
EB-8	AGGGGAGTCTGC [SEQ ID NO:40]	12	FG-3
GP-3	CTGTTTGCCGACA [SEQ ID NO: 41]	13	VH-4
GP-4	TATCGCCATCCAG [SEQ ID NO: 42]	13	VH-4
EB-9	ATGATCGGGGTAGT [SEQ ID NO: 43]	14	FG-4
EB-10	AGAGACCTTAAGGATA [SEQ ID NO:44]	16	FG-4
NG-1	ATTCCAGAACCACGG [SEQ ID NO:45]	15	ON-3
NG-2	TTCCAGAACCACG [SEQ ID NO:46]	13	ON-3
NG-3	TCCAGAACCAC [SEQ ID NO:47]	11	ON-4
GP-5	GTTCTTGGCGCAG [SEQ ID NO: 48]	13	ON-4
GP-6	TTCCTGGCGCAG [SEQ ID NO: 49]	12	ON-4

**Table 5**  
**Capture Sequence Probes for HSV-2**

Probe	Sequence	Size (bp)	Location within HSV-2
NF-1	GCCCGCGCCGCCAGCACTACTTTC [SEQ ID NO:50]	24	41610-41587
FG-1	AAACGTTGGGAGGTGTGTGCGTCATCCTG GAGCTA [SEQ ID NO:51]	35	48200-48234
LE-3	GACCAAAACCGAGTGAGGTTCTGTGT [SEQ ID NO:52]	26	48732-48757
NF-2	AAACGTTGGGAGGTGTGTGCGTCA [SEQ ID NO:53]	24	48200-48223
RA-3	TGCTCGTCACGAAGTCACTCATG [SEQ ID NO:54]	23	22756-22734

ON-2	CATTACTGCCCCGCACCGGACC [SEQ ID NO:55]	21	23862-23842
LE-1	GCCGTGGTGTTCCTGAACACCAGG [SEQ ID NO:56]	24	27666-27643
LE-4	AGTCAGGGTTGCCCCGACTTCGTCAC [SEQ ID NO:57]	25	22891-22867
NF-3	CAGGCGTCCTCGGTCTCGGGCGGGGC [SEQ ID NO:58]	26	32847-32822
NF-4	CCCACGTCACCGGGGGCCCC [SEQ ID NO:59]	20	26743-26724
LE-2	GCCGGTCGCGTGCGACGCCAAGGC [SEQ ID NO:60]	25	33130-33106
SG-3	CCGACGCGTGGGTATCTAGGGGGTCG [SEQ ID NO: 61]	26	90559 – 90534
SG-4	CGGGACGGCGAGCGGAAAGTCAACGT [SEQ ID NO:62]	26	86194 – 86169

**Table 6****Blocker Probes for HSV-2**

Probe Name	Sequence	Size (bp)	Capture Probe to which it hybridizes
HX-4	GGCGCGGGC [SEQ ID NO:63]	9	NF-1
HX-5	GAAAGTAGTGCTGGC [SEQ ID NO:64]	15	NF-1
GP-7	TGCTGGCGGCG [SEQ ID NO:65]	11	NF-1
AZ-3	ACACCTCCCAACG [SEQ ID NO:66]	13	FG-1
AZ-4	CTCCAGGATGACG [SEQ ID NO:67]	13	FG-1
GR-1	TCGGTTTTGGTC [SEQ ID NO:68]	12	LE-3
GR-2	ACACAGAACCTCA [SEQ ID NO:69]	13	LE-3
GP-8	CACACACCTCCCA [SEQ ID NO:70]	13	NF-2
BR-10	CGACCCCCTAGATA [SEQ ID NO:71]	14	SG-3
BR-11	CCACGCGTCGG [SEQ ID NO:72]	11	SG-3
HX-6	ACGTTGACTTTCCGC [SEQ ID NO:73]	15	SG-4
BR-15	CGCCGTCCCG [SEQ ID NO:74]	10	SG-4



**Table 7****Capture Sequence Probes for HPV**

Probe	Sequence	Size (bp)	HPV Type and Sequence Location
ZL-1	GTACAGATGGTACCGGGGTTGTAGAAGTATCTG [SEQ ID NO:75]	33	HPV16 5360-5392
ZL-4	CTGCAACAAGACATACATCGACCGGTCCACC [SEQ ID NO:76]	31	HPV16 495-525
DP-1	GAAGTAGGTGAGGCTGCATGTGAAGTGGTAG [SEQ ID NO:77]	31	HPV16 5285-5315
DP-4	CAGCTCTGTGCATAACTGTGGTAACTTTCTGGG [SEQ ID NO:78]	33	HPV16 128-160
SH-1	GAGGTCTTCTCCAACATGCTATGCAACGTCCTG [SEQ ID NO:79]	33	HPV31 505-537
SH-4	GTGTAGGTGCATGCTCTATAGGTACATCAGGCC [SEQ ID NO:80]	33	HPV31 5387-5419
VS-1	CAATGCCGAGCTTAGTTCATGCAATTTCCGAGG [SEQ ID NO:81]	33	HPV31 132-164
VS-4	GAAGTAGTAGTTGCAGACGCCCTAAAGGTTGC [SEQ ID NO:82]	33	HPV31 5175-5207
AH-1	GAACGCGATGGTACAGGCACTGCAGGGTCC [SEQ ID NO:83]	30	HPV18 5308-5337
AH-2	GAACGCGATGGTACAGGCACTGCA [SEQ ID NO:84]	24	HPV18 5314-5337
AL-1	ACGCCCACCCAATGGAATGTACCC [SEQ ID NO:85]	24	HPV18 4451-4474
PA-4	TCTGCGTCGTTGGAGTCGTTCTGTCTGCTC [SEQ ID NO:86]	32	HPV18 535-566
18-1AB	(TTATTATTA)CTACATACATTGCCGCCATGTTCCCA [SEQ ID NO:87]	36	HPV18 1369-1395
18-2AB	(TTATTATTA)TGTTGCCCTCTGTGCCCCCGTTGTCTATAGCCTCCGT [SEQ ID NO:88]	46	HPV18 1406-1442
18-3AB	(TTATTATTA)GGAGCAGTGCCCAAAAGATTAAAGTTTGC [SEQ ID NO:89]	38	HPV18 7524-7552
18-4AB	(TTATTATTA)CACGGTGCTGGAATACGGTGAGGGGTG [SEQ ID NO:90]	37	HPV18 3485-3512
18-5AB	(TTATTATTA)ACGCCCACCCAATGGAATGTACCC [SEQ ID NO:91]	33	HPV18 4451-4474
18-6AB	(TTATTATTA)ATAGTATTGTGGTGTGTTTCTCACAT [SEQ ID NO:92]	35	HPV18 81-106
18-7AB	(TTATTATTA)GTTGGAGTCGTTCTGTCTGTG [SEQ ID NO:93]	30	HPV18 538-558
18-8AB	(TTATTATTA)CGGAATTTCAATTTTGGGGCTCT [SEQ ID NO:94]	31	HPV18 634-655
PE-1	GCTCGAAGGTCGTCTGCTGAGCTTTCTACTACT [SEQ ID NO:95]	33	HPV18 811-843
PZ-2	GCGCCATCCTGTAATGCACTTTTCCACAAAGC [SEQ ID NO:96]	32	HPV45 77-108
PZ-5	TAGTGCTAGGTGTAGTGGACGCAGGAGGTGG [SEQ ID NO:97]	31	HPV45 5295-5325
CS-1	GGTCACAACATGTATTACACTGCCCTCGGTAC [SEQ ID NO:98]	32	HPV45 500-531

Probe	Sequence	Size (bp)	HPV Type and Sequence Location
CS-4	CCTACGTCTGCGAAGTCTTTCTTGCCGTGCC [SEQ ID NO:99]	31	HPV45 533-563
PF-1	CTGCATTGTCACACTACTATCCCCACCACTACTTTG [SEQ ID NO:100]	34	HPV45 1406-1439
PF-4	CCACAAGGCACATTCATACATACACGCACGCA [SEQ ID NO:101]	32	HPV45 7243-7274
PA-1	GTTCTAAGGTCTCTGCCGAGCTCTCTACTGTA [SEQ ID NO:102]	33	HPV45 811-843
45-5AB	(TTATTATTA)TGCGGTTTTGGGGGTCGACGTGGA GGC [SEQ ID NO:103]	36	HPV45 3444-3470
45-6AB	(TTATTATTA)AGACCTGCCCCCTAAGGGTACATA GCC [SEQ ID NO:104]	36	HPV45 4443-4469
45-8AB	(TTATTATTA)CAGCATTGCAGCCTTTTTGTTACT TGCTTGTAATAGCTCC [SEQ ID NO:105]	49	HPV45 1477-1516
45-9AB	(TTATTATTA)ATCCTGTAATGCACTTTCCACAA A [SEQ ID NO:106]	34	HPV45 79-103
45-10AB	(TTATTATTA)GCCTGGTCACAACATGTATTAC [SEQ ID NO:107]	31	HPV45 514-535
45-11AB	(TTATTATTA)CAGGATCTAATTCATTCTGAGGTT [SEQ ID NO:108]	33	HPV45 633-656
ON-1	TGCGGTTTTGGGGGTCGACGTGGAGGC [SEQ ID NO:109]	27	HPV45 3444-3470

\* Sequences in parentheses are "tail" sequences not directed at HSV.

**Table 8**

**Blocker Probes For HPV**

Probe	Sequence	Size (bp)	Capture Probe to which it hybridizes
PV-FD-1	GCCTCCACGTCGAC [SEQ ID NO:110]	14	ON-1/45-5AB
PV-FD-2	CCCCAAAACCG [SEQ ID NO:111]	11	ON-1/45-5AB
PV-FD-3	GGTACATTCCATTGGG [SEQ ID NO:112]	16	18-5AB/AL-1
PV-FD-4	TGGGCGTTAATAATAA [SEQ ID NO:113]	16	18-5AB
AH-3	ACCATCGCGTTC [SEQ ID NO:114]	12	AH-2
AH-4	GGACCCTGCAGTGC [SEQ ID NO:115]	14	AH-1
AH-5	CTGTACCATCGCGTT 3' [SEQ ID NO:116]	15	AH-1
AH-6	TGCAGTGCCTGT [SEQ ID NO:117]	12	AH-2
PZ-1	CCACCTCCTGCGT [SEQ ID NO:118]	13	PZ-5
PZ-3	ATTACAGGATGGCGC [SEQ ID NO:119]	15	PZ-2
PZ-4	GCTTTGTGGAAGTG [SEQ ID NO:120]	16	PZ-2
PZ-6	CCACTACCTAGCACTA [SEQ ID NO:121]	18	PZ-5
ZL-2	CAGATACTTCTACAACC [SEQ ID NO:122]	17	ZL-1
ZL-3	CCGGTACCATCTGTAC [SEQ ID NO:123]	16	ZL-1
ZL-5	GGTGGACCGGTCG [SEQ ID NO:124]	13	ZL-4
ZL-6	ATGTATGTCTTGTTCAG [SEQ ID NO:125]	18	ZL-4
DP-2	CTACCACTTCACATGC [SEQ ID NO:126]	16	DP-1
DP-3	AGCCTCACCTACTTC [SEQ ID NO:127]	15	DP-1

Probe	Sequence	Size (bp)	Capture Probe to which it hybridizes
DP-5	CCCAGAAAGTTACCAC [SEQ ID NO:128]	16	DP-4
DP-6	AGTTATGCACAGAGCT [SEQ ID NO:129]	16	DP-4
SH-2	CAGGACGTTGCATAGC [SEQ ID NO:130]	16	SH-1
SH-3	ATGTTGGAGAAGACCTC [SEQ ID NO:131]	17	SH-1
SH-5	GGCCTGATGTACCTATA [SEQ ID NO:132]	17	SH-4
SH-6	GAGCATGCACCTACAC [SEQ ID NO:133]	16	SH-4
VS-2	CTCGGAAATTGCATG [SEQ ID NO:134]	15	VS-1
VS-3	AACTAAGCTCGGCATT [SEQ ID NO:135]	16	VS-1
VS-5	GCAACCTTTAGGGG [SEQ ID NO:136]	14	VS-4
VS-6	CGTCTGCAACTACTACTTC [SEQ ID NO:137]	19	VS-4
CS-2	GTACCGAGGGCAGT [SEQ ID NO:138]	14	CS-1
CS-3	GTAATACATGTTGTGACC [SEQ ID NO:139]	18	CS-1
CS-5	GGCACGGCAAGAAA [SEQ ID NO:140]	14	CS-4
CS-6	GACTTCGACAGCTAGG [SEQ ID NO:141]	17	CS-4
PF-2	CAAAGTAGTGGTGGG [SEQ ID NO:142]	15	PF-1
PF-3	GATAGTAGTGACAATGCAG [SEQ ID NO:143]	19	PF-1
PF-5	TGCGTGCGTGTATGTA [SEQ ID NO:144]	16	PF-4
PF-6	TGAATGTGCCTTGTGG [SEQ ID NO:145]	16	PF-4
PE-2	AGTAGTAGAAAGCTCAGC [SEQ ID NO:146]	18	PE-1
PE-3	AGACGACCTTCGAGC [SEQ ID NO:147]	15	PE-1
PA-2	TACAGTAGAGAGCTCGG [SEQ ID NO:148]	17	PA-1
PA-3	CAGAGGACCTTAGAAC [SEQ ID NO:149]	16	PA-1
PA-5	GAGCACGACAGGAACG [SEQ ID NO:150]	16	PA-4
PA-6	ACTCCAACGACGCAGA [SEQ ID NO:151]	16	PA-4

### **Example 3**

#### **Effect of the Extent of Biotin Labeling on Capture Efficiency**

Tests were conducted to determine the optimal number of biotin labels per capture sequence probe for TSHC detection. The general TSHC method described in Example 1 was employed. The capture efficiency of capture sequence probe F15R labelled with one, two, or three biotins, measured by signal to noise ratio (S/N), were tested. The signal sequence probe employed was H19. As shown in Table 9, two biotins per capture sequence probe were sufficient for optimal capture efficiency. Greater than a 50% increase in S/N was observed using capture sequence probe with two biotin labels compared to the single biotin labeled capture sequence probe. The addition of a third biotin label to the capture sequence probe resulted in a decrease in S/N relative to the two-biotin labeled capture sequence probe.

**Table 9****Effect of the Extent of Biotin Labeling on Capture Efficiency**

<b># Biotins</b>	<b><i>HSV-1/well</i></b>	<b><i>RLU</i></b>	<b><i>CV</i></b>	<b><i>S/N</i></b>
One	0	54	3%	1.0
One	$4.5 \times 10^3$	236	2%	4.4
One	$4.5 \times 10^4$	1861	3%	34.5
One	$4.5 \times 10^5$	15633	7%	289.5
Two	0	46	3%	1.0
Two	$4.5 \times 10^3$	296	10%	6.4
Two	$4.5 \times 10^4$	2558	1%	55.6
Two	$4.5 \times 10^5$	23369	4%	508.0
Three	0	44	22%	1.0
Three	$4.5 \times 10^3$	243	6%	5.5
Three	$4.5 \times 10^4$	1820	2%	51.4
Three	$4.5 \times 10^5$	18581	8%	422.3

**Example 4****Effect of the Distance between the CSP and the SSP  
Target Sites on Capture Efficiency**

The effect of the distance between capture sequence probe (CSP) and signal sequence probe (SSP) hybridization sites on a HSV-1 target nucleic acid on capture efficiency was evaluated. CSPs that hybridize to HSV-1 nucleic acid sequences which are located 0.2kb, 3kb, 18kb, 36kb and 46kb from the site of SSP hybridization were tested. The general TSHC method described in Example 1 was employed. The capture efficiencies were 100%, 50%, 30%, 19% and 7%, respectively (Table 10). A steady decline in relative capture efficiencies was observed as the distance increased from 0.2 Kb to 46 Kb.

**Table 10****Effect of Distance between Target Sites on Capture Efficiency**

<u>CSP</u>	<u>SSP</u>	<u>Distance Between Target Site</u>	<u>Relative Capture Efficiency</u>
BRH19	H19	0.2 Kb	100%
F15R	H19	3 Kb	50%
F6R	RH5B	18 Kb	30%
F15R	RH5B	36 Kb	19%
F6R	H19	46 Kb	7%

**Example 5****Effect of Fused Capture Sequence Probe on TSHC Detection of HSV-1**

The binding capacity of streptavidin plates was determined to be approximately 2 pmoles of doubly-biotinylated CSPs per well. Since the CSPs are doubly biotin-labeled, a maximum of 8 CSPs (2 CSPs per SSP) is preferred in order not to exceed the binding capacity of the wells. Any increase in biotin-labeled capture sequence probe above the stated capacity resulted in a decrease in signal, the so-called "hook effect." In order to avoid this "hook effect" and still permit the use of greater than four SSP-CSP combinations, the effect of synthesizing oligonucleotides that contained the sequences of two CSPs fused together (5' and 3' sites) was tested. The fused capture sequence probes may function independently to drive hybridization to the unique target sites. In another embodiment, the fused probes may bind to two target sites with the second hybridization favored, since it is essentially a uni-molecular reaction with zero order kinetics once the probe has hybridized to the first site. The hybridization may be determined by one or both mechanisms. Previous experiments showed that two CSPs, VH3 and NC-1, when used together, gave approximately twice the S/N as the individual CSPs. Unfused capture sequence probes VH-3 and NC-1 were used at 2.5 pmoles/ml each for a total concentration of 5 pmoles/ml, fused probe VH-4 (fusion of VH-3 and NC-1) was used at 2.5 pmole/ml. As shown in Table 11, the fused probe was as effective as the combination of the two unfused probes. Therefore, TSHC detection using fused capture sequence probes permits the number of nucleic acid sequences targeted by the signal sequence probe to

be at least doubled without exceeding the plate biotin-binding capacity. The experiment also demonstrates the lack of cross-reactivity of HSV-2 at  $10^7$  genomes as shown by the S/N less than 2.0.

**Table 11**

**Comparison of Fused v. Unfused Capture Sequence Probes  
in TSHC Detection of HSV-1**

<u>SSP</u>	<u>CSP</u>	<u>Viral Particles/ml</u>	<u>RLU</u>	<u>CV</u>	<u>S/N</u>
RH5B	VH-3, NC-1	0	94	14%	1.0
RH5B	VH-3, NC-1	$10^4$ HSV-1	164	5%	1.7
RH5B	VH-3, NC-1	$10^5$ HSV-1	1003	4%	10.7
RH5B	VH-3, NC-1	$10^7$ HSV-2	125	6%	1.3
RH5B	VH-4 (fused)	0	97	10%	1.0
RH5B	VH-4 (fused)	$10^4$ HSV-1	181	3%	1.9
RH5B	VH-4 (fused)	$10^5$ HSV-1	1070	2%	11.0
RH5B	VH-4 (fused)	$10^7$ HSV-2	140	5%	1.4

**Example 6**

**Capture Efficiency of Various CSPs and SSPs in TSHC Detection of HSV-1**

The capture efficiency of capture sequence probes (CSPs) for each of the four HSV-1 specific signal sequence probes (SSPs), H19, RH5B, RH3 and R10, in the detection of HSV-1 by TSHC were evaluated. The criteria used for designing the capture sequence probes were: 1) the CSP hybridization site is within 1 kb either 5' or 3' of the SSP hybridization site on the HSV-1 nucleic acid sequence, preferably within 0.5 kb; and 2) the CSPs contain sequences that are unique to HSV-1, with no stretches of sequence homology to HSV-2 greater than 10 bases. The CSPs were designed to target the 5' and 3' regions adjacent to the SSP hybridization site, preferably with a 5' CSP and a 3' CSP for each SSP. The Omega software (Oxford Molecular Group, Campbell, CA) was instrumental in the identification of such sites. The melting temperature ( $T_m$ ) of the CSPs was designed to be between  $70^\circ\text{C}$  to  $85^\circ\text{C}$ , to conform to the  $70^\circ\text{C}$  to  $75^\circ\text{C}$  hybridization temperature used in Hybrid Capture II (HCII) assay for HSV (Digene). The general TSHC method described in Example 1 was employed. Eleven CSPs (which bind to 6 different sites) for H19, six CSPs (which bind to three unique sites) for RH5B, six CSPs (which bind to six unique sites) for RH3, and two

CSPs for R10 were tested. As shown in Table 12, efficient capture sequence probes were found for signal sequence probes H19, RH5B and R10.

**Table 12**

**CSPs and SSPs for TSHC Detection of HSV-1**

SSP	CSP	Cap%	SSP	CSP	Cap%	SSP	CSP	Cap%
R10	ON-3	100%	RH5B	TS-1	50%	H19	HZ-1	50%
R10	ON-3	80%	RH5B	NC-1	75%	H19	HZ-2	20%
			RH5B	VH-4	130%	H19	ZD-1	40%
			RH5B	TS-2	25%	H19	ZD-2	20%
			RH5B	VH-3	50%	H19	BRH19	70%
						H19	VH-2	70%
						H19	F15R	25%

**Example 7**

**Capture Efficiency of Various CSPs and SSPs in TSHC Detection of HSV-2**

The capture efficiency of capture sequence probes (CSPs) for each of the four HSV-2 specific signal sequence probes (SSPs), E4A, E4B, Ei8, and i8, in the detection of HSV-2 by TSHC were evaluated. HSV-2 specific capture sequence probes (CSPs) were designed based on the same criteria as the HSV-1 CSPs except for the requirement that they be HSV-2 specific. Four CSPs for E4A, three CSPs for E4B, and two CSPs each for Ei8 and i8 were tested. The general TSHC method described in Example 1 was employed. As shown in Table 13, efficient capture sequence probes were found for i8 and Ei8.

**Table 13****CSPs and SSPs for TSHC Detection of HSV-2**

SSP	CSP	Cap%	SSP	CSP	Cap%
I8	NF-1	100%	Ei8	NF-2	50%
			Ei8	LE-3	45%

**Example 8****Effect of Blocker Probes on HSV-1 and HSV-2 Detection**

In an attempt to reduce cross-reactivity of TSHC while allowing the capture step to take place at room temperature, methods using blocker probes were developed. Blocker probes comprise sequences that are complementary to the capture sequence probes (CSPs) used for detection. These experiments were designed to prevent non-specific hybridization of the CSPs to non-targeted nucleic acids present in the sample under the lower stringency conditions, a situation often encountered during the room temperature capture step.

In one method, blocker probes that are complementary to the full length or nearly the full length of the capture sequences probe were used. The blocker probes were added to the reaction mixture in 10-fold excess relative to the CSP after hybridization of the CSP and the SSP to the target DNA molecule has occurred. Since the blocker probes have similar melting temperature as the CSPs, the CSPs were hybridized to the target nucleic acids first to prevent hybridization of the blocker probes to the CSPs before the hybridization of the CSPs to the target nucleic acids occurred. As shown in Table 14, the addition of the blocker probes resulted in a dramatic reduction in cross-reactivity while these probes had no effect on the sensitivity of HSV-1 detection. The S/N for the detection of cross-reactive HSV-2 ( $10^7$  viral particles/ml) decreased from 5.0 to 0.8 when the blocker probes were used.

In another method, blocker probes that are complementary to only a portion of the CSPs and are shorter than the CSPs were used. The blocker probes were designed to have melting temperatures above room temperature but at least  $10^{\circ}\text{C}$  below the hybridization temperature of CSPs to the target nucleic acids. Since these blocker probes hybridize to the CSPs at temperature below the CSP hybridization



temperature to the target nucleic acids, the blocker probes may be added to the reaction at the same time as the CSP and SSP without effecting the hybridization efficiency of the CSPs to the target nucleic acid. These shorter blocker probes function during the room temperature capture step by hybridizing to the CSPs at the lower temperatures that are encountered during the room temperature capture step. As shown in Table 15, the addition of either single or paired shorter blocker probes in 100-fold excess relative to the CSPs resulted in a dramatic reduction in cross-reactivity but had no effect on sensitivity of HSV-1 detection. The S/N for detecting cross-reactive HSV-2 ( $10^7$  viral particles/ml) without the blocker probes was 10.6, but was reduced to less than or equal to 1.5 with the addition of the blocker probes.

Therefore, both methods utilizing blocker probes provide a substantial reduction in cross-reactivity. The second method utilizing blocker probes with lower melting temperature may be preferred because the addition of blocker probes at the same time as the capture sequence probe eliminates the need for an extra step for the detection method.

**Table 14**

**Effect of Blocker Probes Added Post Capture probe hybridization on TSHC**

<u>SSP</u>	<u>CSP</u>	<u>100x Blocker Probe</u>	<u>Viral Particles/ml</u>	<u>RLU</u>	<u>CV</u>	<u>S/N</u>
H19	HZ-1	None	0	66	7%	1.0
H19	HZ-1	None	$10^5$ HSV-1	246	5%	3.7
H19	HZ-1	None	$10^6$ HSV-1	1998	2%	30.3
H19	HZ-1	None	$10^7$ HSV-2	327	2%	5.0
H19	HZ-1	ZD-3	0	60	3%	1.0
H19	HZ-1	ZD-3	$10^5$ HSV-1	267	4%	4.5
H19	HZ-1	ZD-3	$10^6$ HSV-1	2316	6%	38.6
H19	HZ-1	ZD-3	$10^7$ HSV-2	49	2%	0.8

**Table 15**

**Effect of Blocker Probes Added Simultaneously with the Capture Probes  
on TSHC Detection of HSV-1**

<u>SSP</u>	<u>CSP</u>	<u>10x Blocker Probe</u>	<u>Viral Particle/ml</u>	<u>RLU</u>	<u>CV</u>	<u>S/N</u>
H19	HZ-1	none	0	38	15%	1.0
H19	HZ-1	none	10 <sup>4</sup> HSV-1	71	2%	1.9
H19	HZ-1	none	10 <sup>5</sup> HSV-1	389	12%	10.2
H19	HZ-1	none	10 <sup>7</sup> HSV-2	401	18%	10.6
H19	HZ-1	NG-4	0	39	8%	1.0
H19	HZ-1	NG-4	10 <sup>4</sup> HSV-1	82	5%	2.1
H19	HZ-1	NG-4	10 <sup>5</sup> HSV-1	411	18%	10.5
H19	HZ-1	NG-4	10 <sup>7</sup> HSV-2	57	15%	1.5
H19	HZ-1	EA-1, EA-2	0	37	0%	1.0
H19	HZ-1	EA-1, EA-2	10 <sup>4</sup> HSV-1	75	8%	2.0
H19	HZ-1	EA-1, EA-2	10 <sup>5</sup> HSV-1	419	8%	11.3
H19	HZ-1	EA-1, EA-2	10 <sup>7</sup> HSV-2	49	5%	1.3
H19	HZ-1	NG-7, NG-8	0	42	10%	1.0
H19	HZ-1	NG-7, NG-8	10 <sup>4</sup> HSV-1	76	3%	1.8
H19	HZ-1	NG-7, NG-8	10 <sup>5</sup> HSV-1	471	5%	11.2
H19	HZ-1	NG-7, NG-8	10 <sup>7</sup> HSV-2	47	9%	1.1

**Example 9****TSHC Detection Reduces Vector Background**

The TSHC assay eliminates the vector contamination problem often associated with the Hybrid Capture II (HC II) detection assay (Digene). As the RNA signal sequence probes used in HC II are generated from linearized vector templates, any remaining unlinearized plasmid DNA results in the production of additional RNA probe sequences specific for vector sequences. In the HC II assay, the RNA/DNA hybrids that form as a result of these read-through transcripts are captured on the antibody coated plates and generate signal. In contrast, in the TSHC method, only those RNA/DNA hybrids that also hybridize to the capture sequence probes are detected. Accordingly, any detection of vector-related sequences is eliminated. Plasmids SK+, pBR322, DgZ and 1066 which were known to be detectable in HSV HC II test (Digene) were tested in the TSHC assay using two RNA signal sequence probes (H19 and RH5b) and two capture sequence probes (VH-2 and VH-4). Identical set of RNA probes were then used in HC II method and the TSHC method for the detection of HSV-1. The general TSHC method described in Example 1 was

employed. As shown in Table 16, while signal to noise ratio in standard HC II ranged from 14 to 48, the signal to noise ratio for the TSHC method was less than 2 for all plasmids tested.

**Table 16**

**Vector Background in TSHC v. HCII Detection**

<u>Method</u>	<u>SSP</u>	<u>CSP</u>	<u>Targets/ml</u>	<u>RLU</u>	<u>CV</u>	<u>S/N</u>
TSHC	H19 + RH5B	VH-2 + VH-4	0	94	6%	1.0
TSHC	H19 + RH5B	VH-2 + VH-4	4 ng pBS SK+	137	7%	1.5
TSHC	H19 + RH5B	VH-2 + VH-4	2 ng pBR322	99	6%	1.1
TSHC	H19 + RH5B	VH-2 + VH-4	4 ng DgX	135	7%	1.4
TSHC	H19 + RH5B	VH-2 + VH-4	4 ng 1066	107	7%	1.1
HC II	H19 + RH5B	None	0	94	9%	1.0
HC II	H19 + RH5B	None	4 ng pBS SK+	4498	3%	48.1
HC II	H19 + RH5B	None	2 ng pBR322	1281	8%	13.7
HC II	H19 + RH5B	None	4 ng DgX	2003	5%	21.4
HC II	H19 + RH5B	None	4 ng 1066	1536	2%	16.4

**Example 10**

**Sensitivity and Specificity of detecting HSV-1 and HSV-2 by TSHC**

The sensitivity and typing discrimination for the TSHC detection of HSV-1 and HSV-2 were assessed using the TSHC described in Example 1. In the HSV-1 TSHC assay, signal sequence probes H19 and RH5B, capture sequence probes HZ-1, VH-2 and VH-4, and blocker probes NG-7, NG-8, GP-3, GP-4, and GP-1 were used. In the HSV-2 TSHC assay, signal sequence probes I8 and Ei8, capture sequence probes NF-1 and NF-2, and blocker probes HX-4, HX-5 and GP-8 were used. HSV-1 and HSV-2 viral particles were diluted to various concentrations using the Negative Control Solution. As shown in Figures 4 and 5, while  $10^4$  copies of the either HSV-1 or HSV-2 (450 copies/well) were detected in the respective assays, there was virtually no detection of the cross-reactive type HSV at concentrations up to and including  $10^8$  copies/ml (4,500,000 copies/well). Thus, the HSV-1 and HSV-2 TSHC assays can

distinguish the two HSV types at a greater than 10,000-fold range of discrimination while maintaining excellent sensitivity (450 VP/well).

The HSV-1 TSHC assay shows a linear range of detection ranging from at least  $2 \times 10^3$  to  $5 \times 10^3$  VP/ml (Table 17). The specificity of the assay is excellent as no cross-reactivity was detected (S/N is less than or equal to 2) in samples containing HSV-2 at a concentration as high as  $2 \times 10^7$  to  $5 \times 10^7$  viral particles/ml. Similarly, the HSV-2 TSHC assay also shows excellent specificity, wherein no cross-reactivity was detected in samples containing HSV-1 at a concentration as high as  $5 \times 10^7$  viral particles/ml (Table 18). Similar results were obtained from TSHC detection of HSV-2 using a dilution series of HSV-2 and HSV-1 viruses (Table 19).

**Table 17**

**Analytical Sensitivity and Specificity of the HSV1 TSHC Assay**

<b>Targets</b>	<b>RLU</b>	<b>S/N</b>
Negative Control	47	1.0
HSV2 @ $5 \times 10^7$ VP/ml	57	1.2
HSV2 @ $2 \times 10^7$ VP/ml	43	0.9
HSV1 @ $5 \times 10^3$ VP/ml	201	4.3
HSV1 @ $2 \times 10^3$ VP/ml	107	2.3

**Table 18**

**Analytical Sensitivity and Specificity of the HSV2 TSHC Assay**

<b>Targets</b>	<b>RLU</b>	<b>S/N</b>
Negative Control	40	1.0
HSV1 @ $5 \times 10^7$ VP/ml	78	2.0
HSV1 @ $2 \times 10^7$ VP/ml	55	1.4
HSV2 @ $5 \times 10^3$ VP/ml	218	5.5
HSV2 @ $2 \times 10^3$ VP/ml	106	2.7

**Table 19****Detection with HSV-2 Probes using HSV-1 and HSV-2 of Different Dilution**

<b>Targets</b>	<b>RLU</b>	<b>S/N</b>
Negative Control	43	1.0
HSV1 @ 5x10 <sup>7</sup> VP/ml	112	2.6
HSV1 @ 2x10 <sup>7</sup> VP/ml	57	1.3
HSV1 @ 1x10 <sup>7</sup> VP/ml	38	0.9
HSV1 @ 1x10 <sup>6</sup> VP/ml	38	0.9
HSV1 @ 1x10 <sup>5</sup> VP/ml	33	0.8
HSV1 @ 1x10 <sup>4</sup> VP/ml	52	1.2
HSV1 @ 1x10 <sup>3</sup> VP/ml	43	1.0
HSV1 @ 1x10 <sup>2</sup> VP/ml	39	0.9
HSV2 @ 1x10 <sup>7</sup> VP/ml	257173	5980.8
HSV2 @ 1x10 <sup>6</sup> VP/ml	28544	663.8
HSV2 @ 1x10 <sup>5</sup> VP/ml	3200	74.4
HSV2 @ 1x10 <sup>4</sup> VP/ml	266	6.2
HSV2 @ 5x10 <sup>3</sup> VP/ml	181	4.2
HSV2 @ 1x10 <sup>3</sup> VP/ml	62	1.4
HSV2 @ 1x10 <sup>2</sup> VP/ml	44	1.0

**Example 11****Clinical Specimen Testing**

A 64-member clinical specimen panel was tested for HSV-1 and HSV-2 using both TSHC and HCII methods. The panel included 15 samples containing known quantities of HSV-1 or HSV-2, and 49 samples known to be negative for HSV-1 and HSV-2 by PCR testing. Accordingly, the 15 positive samples were “Expected” to test positive in both the HCII and TSHC assays, and the 49 negative samples were “Expected” to test negative in both the HCII and TSHC tests.

The general TSHC method described in Example 1 was employed. The results using the HCII method and the TSHC method are shown in Tables 20 and 21, respectively. Of the 49 samples “Expected” to yield negative result, 5 samples tested positive and 44 samples tested positive using the HCII method. In comparison, all 49

samples tested negative using the TSHC method. Therefore, the TSHC method is superior in specificity to the HCII method in the detection of HSV-1 and HSV-2.

**Table 20**

**Observed vs. Expected Results for HCII Detection of HSV1 and HSV2**

HCII Result	Expected Result	
	Positive	Negative
Positive	15	5
Negative	0	44
Total	15	49

**Table 21**

**Observed vs. Expected Results for TSHC Detection of HSV1 and HSV2**

TSHC Result	Expected Result	
	Positive	Negative
Positive	14	0
Negative	1	49
Total	15	49

**Example 12**

**Effect of Combining Probes in TSHC Detection of HSV**

The effect of combining HSV-1 specific signal sequence probe and capture sequence probe sets on HSV-1 detection was assessed. TSHC detection of HSV-1 and HSV-2 cross-reactivity was performed separately with two different sets of RNA signal sequence probe /biotinylated capture sequence probe combinations (Set #1: H19 plus HZ-1; and Set #2: RH5b plus the TS-1 and TS-2). TSHC was also performed with both RNA signal sequence probe/biotinylated capture sequence probe sets combined to assess the effect of combining the two probe sets on sensitivity and cross-reactivity. The general TSHC method described in Example 1 was employed. The results shown in Table 22 clearly demonstrate an additive effect of combining the two probe sets for HSV-1 detection with no apparent increase in HSV-2 cross-reactivity.

**Table 22****Sensitivity is Improved by Combining HSV-1 Specific CSPs and SSPs**

Capture Sequence Probes	Signal Sequence Probes	VP/ml	RLU	CV	S/N
HZ-1	H19	0	60	3%	1.0
HZ-1	H19	10 <sup>5</sup> HSV-1	267	4%	4.5
HZ-1	H19	10 <sup>6</sup> HSV-1	2316	6%	38.9
HZ-1	H19	10 <sup>7</sup> HSV2	49	2%	0.8
TS-1, TS-2	RH5B	0	78	6%	1.0
TS-1, TS-2	RH5B	10 <sup>5</sup> HSV-1	291	6%	3.8
TS-1, TS-2	RH5B	10 <sup>6</sup> HSV-1	2368	11%	30.6
TS-1, TS-2	RH5B	10 <sup>7</sup> HSV2	75	11%	1.0
HZ-1, TS-1, TS-2	H19, RH5B	0	70	12%	1.0
HZ-1, TS-1, TS-2	H19, RH5B	10 <sup>5</sup> HSV-1	457	10%	6.5
HZ-1, TS-1, TS-2	H19, RH5B	10 <sup>6</sup> HSV-1	4263	1%	60.9
HZ-1, TS-1, TS-2	H19, RH5B	10 <sup>7</sup> HSV2	67	6%	1.0

**Example 13****TSHC Detection of HPV18 and HPV45**

The relative sensitivity and specificity of TSHC and HCII detection of Human Papillomavirus 18 (HPV18) and Human Papillomavirus 45 (HPV45) was compared. Previous studies have established HPV45 as the most cross-reactive HPV type to HPV18, and conversely, HPV18 as the most cross-reactive HPV type to HPV45. In this study, the ability of the two methods to detect HPV18 and HPV45 was assessed using HPV18 and HPV45 plasmid DNA.

Capture sequence probes (CSPs) for each of the four Human Papillomavirus types: HPV16, HPV18, HPV31, and HPV45, were designed. The criteria used for designing the capture sequence probes were: 1) the CSP hybridization sites do not overlap with the SSP sites; 2) the CSPs contain sequences unique to one HPV type with no stretches of sequence homology to other HPV types greater than 12 bases; and 3) the CSPs are of sufficient length so as to be capable of hybridizing efficiently at 70°C.

The blocker probes for each CSP were designed such that they could be added simultaneously with the CSP during hybridization to the target nucleic acid. The blocker probes have a melting temperature of at least 37°C but no higher than 60°C, as calculated by the Oligo 5.0 program (National Biosciences, Inc., Plymouth, MN). Two blocker probes were used for each capture oligonucleotide to maximize the blocker effect during the room temperature plate capture step. It was also desired that the blocker probes for each CSP have similar melting temperatures.

CSPs for each of the HPV types were tested for relative capture efficiency and cross-reactivity to other HPV types. CSPs that provided the best combination of sensitivity and low cross-reactivity were used for the detection of HPV using TSHC.

In TSHC and HCII detection of HPV18, HPV18 DNA was used at a concentration of 10 pg/ml. HPV45, used for cross-reactivity testing, was used at 4 ng/ml. The general TSHC method described in Example 1 was employed. As shown in Table 23, a signal to noise ratio of 16.9 was obtained for TSHC detection of HPV18 compared to a ratio of 7.6 obtained for HCII detection of HPV18. On the other hand, cross-reactivity with HPV45 was significantly reduced using the TSHC method (S/N of 1.3 for TSHC compared to S/N of 393.3 for HCII). The results clearly show that compared to the HCII method, the TSHC method for the detection of HPV18 was superior in both sensitivity and specificity. Results obtained in experiments comparing TSHC and HCII detection of HPV45 demonstrate that the TSHC method for the detection of HPV45 is superior in both sensitivity and specificity (Table 24).



**Table 23****TSHC Detection of HPV 18**

Method	Target	SSP	CSP	S/N
TSHC	0	18L1	18-7L	1.0
	HPV18 (10 pg/ml)	18L1	18-7L	16.9
	HPV45 (4 ng/ml)	18L1	18-7L	1.3
HC II	0	18L1	none	1.0
	HPV18 (10 pg/ml)	18L1	none	7.6
	HPV45 (4 ng/ml)	18L1	none	393.3

**Table 24****TSHC Detection of HPV 45**

Method	Target	SSP	CSP	S/N
TSHC	0	45L1	ON-1	1.0
	HPV45 (10 pg/ml)	45L1	ON-1	8.4
	HPV18 (4 ng/ml)	45L1	ON-1	1.6
HC II	0	45L1	none	1.0
	HPV45 (10 pg/ml)	45L1	none	8.2
	HPV18 (4 ng/ml)	45L1	none	494.0

**Example 14****Target-Specific Hybrid Capture-Plus Assay Protocol**

Hepatitis B Virus (HBV) was used as the model system for the development of the target-specific hybrid capture-plus (TSHC-plus) assay for the detection of target nucleic acids.

The hybridization in the TSHC-plus method (Fig. 6A-6D) may be performed in a single step. In the one-step method, CSPs, SSPs containing pre-hybridized DNA-RNA duplex, bridge probes (Fig. 6B-6D), and blocker probes are added simultaneously to the target nucleic acids. If hybridization is performed in two steps, CSPs, SSPs without pre-hybridized DNA-RNA duplex, bridge probes and blocker probes are first hybridized to the target nucleic acid. Oligonucleotide probes

complementary to the single stranded nucleic acid sequence in the SSP are then added to the reaction to form the DNA-RNA duplexes. The hybrids are then detected using anti-RNA/DNA antibody as described in Example 1.

Experiments were carried out to detect HBV using TSHC-plus (Examples 15-18). The method shown in Figure 6A was used. Human hepatitis B virus (HBV adw2) plasmid DNA of known concentration (Digene Corp) was diluted using HBV negative Sample Diluent (Digene). Various dilutions were made and aliquoted into individual tubes. The negative Sample Diluent was used as a negative control. A half volume of the Denaturation Reagent 5100-0431 (Digene) was added to the test samples. Test samples were incubated at 65° C for 45 minutes to denature the nucleic acids in the samples.

Following denaturation of the HBV sample, a hybridization solution containing capture sequence probes (CSPs), blocker probes, signal sequence probe comprising a M13 DNA/M13 RNA duplex and a single-stranded DNA sequence capable of hybridizing to HBV sequences was added to the samples, and incubated at 65° C for 1-2 hours. Alternatively, the denatured samples were incubated for 1 hour with a hybridization solution containing capture sequence probes (CSPs), blocker probes and M13 DNA plasmid containing HBV complementary sequences for 1 hour. Following the incubation, M13 RNA was added to the reaction and the incubation was continued for an additional hour at 65° C.

Tubes containing reaction mixtures were cooled at room temperature for 5 minutes and aliquots were taken from each tube and transferred to individual wells of a 96-well streptavidin plate (Digene). The plates were shaken at 1100 rpms for 1 hour at room temperature. The solution was then decanted and the plates were washed four times with SNM wash buffer (Digene). The alkaline-phosphatase anti-RNA/DNA antibody DR-I (Digene) was added to each well and incubated for 30 minutes at room temperature. The DR-1 (Digene) was then decanted and the plates were washed four times with SNM wash buffer (Digene). Following removal of the residual wash buffer, luminescent substrate (CDP-Star, Tropix Inc.) was added to each well and incubated for 15 minutes at room temperature. Individual wells were read on a plate luminometer to obtain relative light unit (RLU) signals.

**Example 15**

The following tables describe the various probes tested in the experiments described in Examples 16-18.

**Table 25****Capture Sequence Probes for HBV**

Probe	Sequence	Size (bp)	Location within HBV	Strand
HBV C1	GCTGGATGTGTCTGCGGCGTTTTATCAT (SEQ ID NO: 152)	28	374-401	Sense
HBV C2	ACTGTTCAAGCCTCCAAGCTGCGCCTT (SEQ ID NO: 153)	27	1861-1877	Sense
HBV C3	ATGATAAAACGCCGCAGACACATCCAGCG ATA (SEQ ID NO: 154)	32	370-401	Anti-sense

**Table 26****HBV/M13 Clones from which SSPs are Prepared**

Clone name	Vector	Cloning site	Insert Size (bp)	Location within HBV
SA1	M13 mp 18	Eco RI, Hind III	35	194-228
SA2	M13 mp 18	Eco RI, Hind III	34	249-282
SA1a	M13 mp 19	Eco RI, Hind III	35	194-228
SA2a	M13 mp 19	Eco RI, Hind III	34	249-282
SA4	M13 mp 19	Eco RI, Hind III	87	1521-1607

**Table 27****HBV Blocker probes**

Probe	Sequence	Size (bp)	CSP to which it hybridizes
B1	ATGATAAAACGCCG (SEQ ID NO: 155)	14	HBV C1
B2	CAGACACATCCAGC (SEQ ID NO: 156)	14	HBV C1
B3	AAGGCACAGCTTG (SEQ ID NO: 157)	13	HBV C2
B4	GAGGCTTGAACAGT (SEQ ID NO: 158)	14	HBV C2
B5	TATCGCTGGATGTGTC (SEQ ID NO: 159)	16	HBV C3
B6	TCGGCGTTTTATCATG (SEQ ID NO: 160)	16	HBV C3

### **Example 16**

#### **Effect of Blocker Probes on TSHC-Plus Detection of HBV**

During room temperature capture step, excess SSP (M13 RNA/HBV-M13 DNA duplex) non-specifically hybridizing to the CSP are immobilized onto the plate which results in high background signals. In an attempt to reduce background signal, blocker probes were employed in TSHC-Plus detection of HBV. The blocker probes were designed to be much shorter than the CSPs so that they are only capable of hybridizing to the capture probes at temperatures well below the hybridization temperatures used in the assay.

Blocker probe sets consisting of two separate oligonucleotides that are complementary to the CSPs were used. The blocker probes were added to the hybridization mixture in 10-fold excess relative to the CSPs. Since the blocker probes are much shorter than the CSPs, they do not hybridize with CSPs at the target hybridization temperature and therefore do not interfere with the hybridization of the CSPs to the target nucleic acids. Following the hybridization of CSP and target nucleic acids, the samples were subjected to a room temperature capture step during which the blocker probes hybridize with excess CSPs, thus preventing them from hybridizing to the SSPs. As shown in Table 28, the use of the blocker probes in the hybridization reaction greatly reduced the background signals of the assay.

**Table 28**

#### **Effect of Blocker Probes on HBV Detection**

<b>Capture Probe</b>	<b>Blocker probe</b>	<b>Background Signal (RLU)</b>
HBV C1	no	17892
HBV C1	B1, B2	424
HBV C2	no	9244
HBV C2	B3, B4	398

### **Example 17**

#### **Effect of the Length of SSP on TSHC-Plus Detection of HBV**

The effect of the length of the DNA sequence inserted into the M13 vector for generating the SSP on TSCH-Plus detection of HBV was studied. A

positive control containing 20 pg/ml of HBV plasmid DNA was used. As shown in Table 29, the use of a longer HBV complementary sequence in the SSP (87 base pairs) resulted in a substantial increase in signal of detection. The effect is unlikely due to sub-optimal hybridization temperature condition since the  $T_m$  of the shorter probes is 15 degree above the hybridization temperature. As the M13 RNA-DNA duplex formed in the SSP may act to partially block the complementary DNA sequence in the probe from hybridizing to the HBV sequences in the target nucleic acids, longer complementary sequences in the SSP may overcome this block.

**Table 29**

**Effect of the Length of the Complementary sequence in the SSP on TSHC-Plus Detection of HBV**

SSP	Size of the HBV Target DNA Sequence in SSP (bp)	$T_m$ of the HBV Target DNA Sequence in SSP	Hybridization temperature	Signal (RLU)
SA1	35	83° C	65° C	1741
SA2	34	80° C	65° C	1857
SA4	87	108° C	65° C	7978

**Example 18**

**TSHC-Plus and HC II Detection of HBV**

The relative sensitivity of TSHC-Plus and HC II (Hybrid Capture II, Digene) detection of HBV was compared. HBV positive standards of three different concentrations were tested in the experiments. As shown in Table 30, the signals obtained using the TSHC-Plus detection method were approximately two-fold higher than those obtained using the HC II detection method.

**Table 30**  
**TSHC-Plus and HC II Detection of HBV\***

Method	Control	Target HBV Concentration		
		10 pg/ml	20 pg/ml	100 pg/ml
HC II	48	2355	4225	21438
TSHC Plus	285	4856	7978	37689

\* Signal measured as relative light unit (RLU)

The above description of various preferred embodiments has been presented for purposes of illustration and description. It is not intended to be exhaustive or limiting to the precise forms disclosed. Obvious modifications or variations are possible in light of the above teachings. The embodiments discussed were chosen and described to provide illustrations and its practical application to thereby enable one of ordinary skill in the art to utilize the various embodiments and with various modifications as are suited to the particular use contemplated. All such modifications and variations are within the system as determined by the appended claims when interpreted in accordance with the breadth to which they are fairly, legally and equitably entitled.

We claim:

1. A method of detecting a target nucleic acid comprising:
  - a) hybridizing a single-stranded target nucleic acid to a capture sequence probe and a signal sequence probe to form double-stranded hybrids between said probes and the target nucleic acid, wherein the capture sequence probe and the signal sequence probe are capable of hybridizing to non-overlapping regions within the target nucleic acid and not being capable of hybridizing to each other;
  - b) adding a blocker probe to the hybridization reaction, wherein said blocker probe hybridizes to excess non-hybridized capture sequence probes;
  - c) capturing the hybrid to form a bound hybrid; and
  - d) detecting the bound hybrid.
2. A method of detecting a target nucleic acid comprising:
  - a) hybridizing a single-stranded target nucleic acid to an immobilized capture sequence probe and a signal sequence probe to form double-stranded hybrids between said probes and the target nucleic acid, wherein the capture sequence probe and the signal sequence probe are capable of hybridizing to non-overlapping regions within the target nucleic acid and not being capable of hybridizing to each other;
  - b) adding a blocker probe to the hybridization reaction, wherein said blocker probe hybridizes to excess non-hybridized capture sequence probes;
  - c) detecting the bound hybrid.
3. The method of claim 1 or 2, wherein the capture sequence probe is modified with at least one ligand.
4. The method of claim 1 or 2, wherein the signal sequence probe is unlabelled.
5. The method of claim 3, wherein the ligand is biotin.

6. The method of claim 5, wherein the capture sequence probe is linear having a 5' and 3' end, wherein both the 5' and the 3' ends are biotinylated.
7. The method of claim 1 or 2, wherein the capture sequence probe and the signal sequence probe hybridize to regions of the target nucleic acid, wherein the regions are less than 3 kilobases apart.
8. The method of claim 1 or 2, wherein the capture sequence probe and the signal sequence probe hybridize to regions of the target nucleic acid, wherein the regions are less than 500 bases apart.
9. The method of claim 1 or 2, wherein the capture sequence probe is a fusion of two or more sequences complementary to different regions of the target nucleic acid or to different target molecules.
10. The method of claim 1 or 2, wherein the double-stranded hybrid formed is a DNA-RNA hybrid.
11. The method of claim 1 or 2, further comprising the step of forming single-stranded DNA prior to the hybridization step.
12. The method of claim 1 or 2, wherein hybridization of the capture sequence probe and the signal sequence probe to the target nucleic acid are performed sequentially.
13. The method of claim 1 or 2, wherein step a) and step b) are performed simultaneously.
14. The method of claim 1 or 2, wherein the blocker probe has lower melting temperature than that of the capture sequence probe.
15. The method of claim 1, wherein the hybrid is captured onto a solid phase.
16. The method of claim 15, wherein the solid phase is coated with streptavidin.
17. The method of claim 15, wherein the solid phase is a microplate.



18. The method of claim 1 or 2, wherein step c) is carried out at room temperature.
19. The method of claim 1 or 2, wherein the bound hybrid is detected using an antibody capable of recognizing a hybrid.
20. The method of claim 19, wherein the hybrid is a DNA-RNA-hybrid.
21. The method of claim 20, wherein the antibody capable of recognizing a DNA-RNA hybrid is labelled with alkaline-phosphatase.
22. A method of detecting a target nucleic acid comprising:
  - a) hybridizing a single-stranded target nucleic acid to a capture sequence probe and a signal sequence probe, wherein the capture sequence probe and the signal sequence probe are capable of hybridizing to non-overlapping regions within the target nucleic acid and not being capable of hybridizing to each other, wherein said hybridization forms an RNA-DNA hybrid between said signal sequence probe and the target nucleic acid; and
  - b) detecting the RNA-DNA hybrid by binding an antibody capable of recognizing the RNA-DNA hybrid to said hybrid, wherein said antibody is detectably labelled.
23. The method of claim 22, further comprising capturing the hybrid formed in step a) to form a bound hybrid.
24. The method of claim 22, wherein the capture sequence probe is modified with at least one ligand.
25. The method of claim 22, wherein the signal sequence probe is unlabelled.
26. The method of claim 24, wherein the capture sequence probe is biotinylated.
27. The method of claim 26, wherein the capture sequence probe is linear having a 5' and a 3' end, wherein both the 5' and the 3' ends are biotinylated.

28. The method of claim 22, wherein the capture sequence probe and the signal sequence probe hybridize to regions of the target nucleic acid, wherein the regions are less than 3 kilobases apart.

29. The method of claim 22, wherein the capture sequence probe and the signal sequence probe hybridize to regions of the target nucleic acid, wherein the regions are less than 500 bases apart.

30. The method of claim 22, further comprising the step of forming single-stranded target DNA prior to the hybridization step.

31. The method of claim 22, wherein hybridizations of the capture sequence probe and the signal sequence probe to the target nucleic acid are performed sequentially.

32. The method of claim 1, wherein the hybrid formed in step a) is captured onto a solid phase.

33. The method of claim 30, wherein the capture step is carried out at room temperature.

34. The method of claim 22, wherein the solid phase is coated with streptavidin.

35. The method of claim 22, wherein the solid phase is a microplate.

36. The method of claim 22, wherein the antibody is labelled with alkaline-phosphatase.

37. The method of claim 20, further comprising adding a blocker probe to the hybridization step, wherein said blocker probe hybridizes to excess non-hybridized capture sequence probes.

38. The method of claim 37, wherein the blocker probes are added to the hybridization reaction following the hybridization of the capture sequence probes to the target nucleic acid.

39. The method of claim 37, wherein the blocker probe has lower melting temperature than that of the capture sequence probe.

40. A method of detecting a target nucleic acid comprising:

a) hybridizing a single stranded target nucleic acid to a capture sequence probe and a signal sequence probe, wherein the capture sequence probe and the signal sequence probe are capable of hybridizing to non-overlapping regions within the target nucleic acid and not being capable of hybridizing to each other, wherein the signal sequence probe comprises a DNA-RNA hybrid region, wherein said hybridization forms a complex; and

b) detecting said complex.

41. The method of claim 40 wherein the capture sequence probe is immobilization on a solid matrix.

42. The method of claim 40 wherein said complex is detected by binding an antibody capable of recognizing the DNA-RNA hybrid region to said region, wherein the antibody is detectably labelled.

43. The method of claim 40 wherein the capture sequence is modified with at least one ligand.

44. The method of claim 43 wherein the ligand is biotin.

45. The method of claim 44 wherein two biotin molecules are attached to the capture sequence probe.

46. The method of claim 40, further comprising adding a blocker probe after the hybridization step, wherein said blocker probe hybridizes to excess non-hybridized capture sequence probe.

47. A nucleic acid probe consisting of a sequence selected from the group consisting of SEQ ID NO:1 through SEQ ID NO:160.

48. The method according to claim 1, wherein the signal sequence probe comprises a DNA-RNA duplex and a single stranded nucleic acid sequence which is capable of hybridizing to the target nucleic acid.

49. The method according to claim 48, wherein the DNA-RNA duplex is a M13 DNA-M13 RNA duplex.

50. A method of detecting a target nucleic acid comprising:

a) hybridizing a single-stranded target nucleic acid to a capture sequence probe, a bridge probe and a signal sequence probe to form double-stranded hybrids between said capture sequence and bridge probes and the target nucleic acid, wherein the capture sequence probe and the bridge probe are capable of hybridizing to non-overlapping regions within the target nucleic acid and not being capable of hybridizing to each other, and the signal sequence probe is capable of hybridizing to the bridge probe and not being capable of hybridizing to the target nucleic acid and the capture sequence probe;

b) adding a blocker probe to the hybridization reaction, wherein said blocker probe hybridizes to excess non-hybridized capture sequence probes;

c) capturing the hybrid to form a bound hybrid; and

d) detecting the bound hybrid.

51. The method according to claim 50, wherein the signal sequence probe comprises a DNA-RNA duplex and a single stranded nucleic acid which is capable of hybridizing to the bridge probe.

52. The method according to claim 51, wherein the DNA-RNA duplex is a M13 DNA-M13 RNA duplex.

53. The method according to claim 51, wherein the DNA-RNA duplex is a hybrid formed between repeat sequences within the signal sequence probe and a nucleic acid molecule having complementary sequences to the repeat sequences.

54. The method according to claim 50, wherein the bridge probe further comprises a poly(A) tail.

55. The method according to claim 54, wherein the signal sequence probe comprises a single stranded poly(dT) DNA sequence which is capable of

hybridizing to the poly(A) tail of the bridge probe, and a DNA-RNA duplex formed between the poly(dT) sequences in the signal sequence probe and a nucleic acid molecule having poly(A) sequences.

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## ABSTRACT

Target-specific hybrid capture (TSHC) provides a nucleic acid detection method that is not only rapid and sensitive, but is also highly specific and capable of discriminating highly homologous nucleic acid target sequences. The method produces DNA-RNA hybrids which can be detected by a variety of methods.

## Target Specific Hybrid Capture

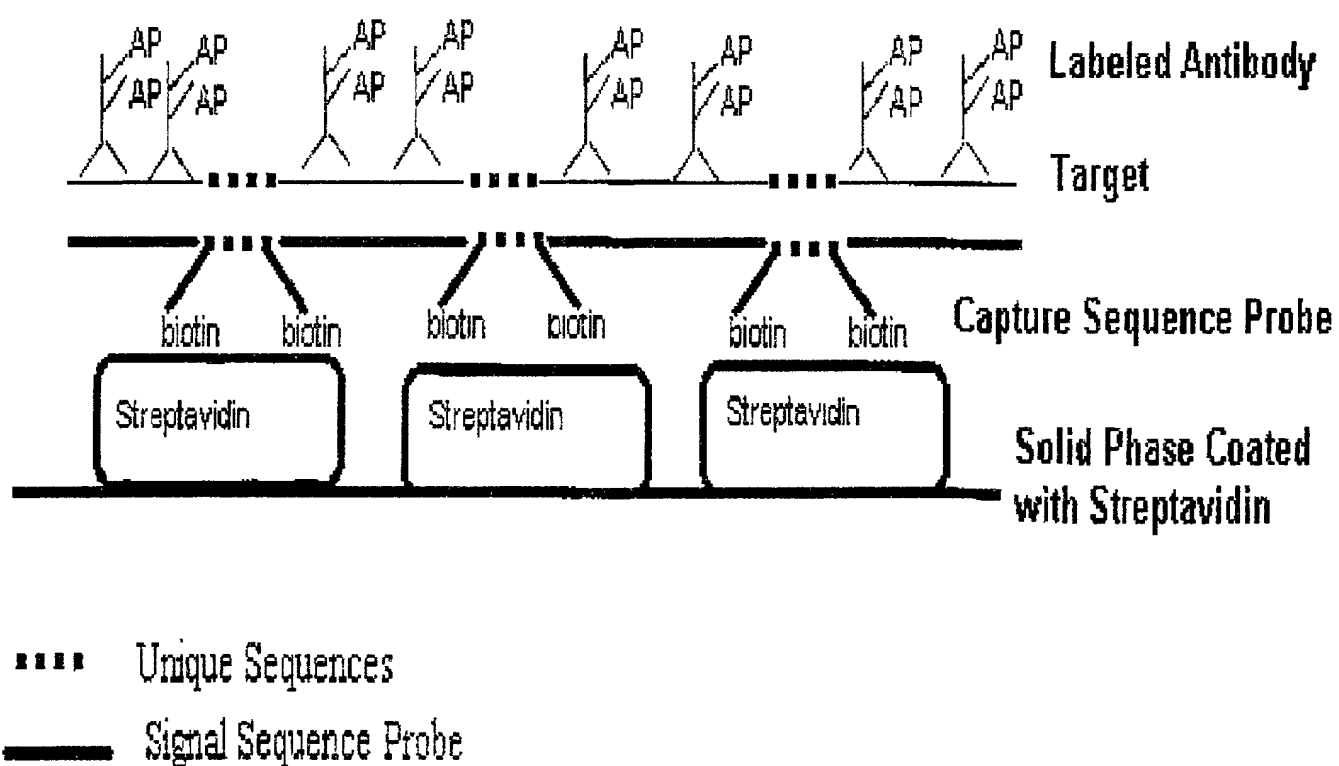
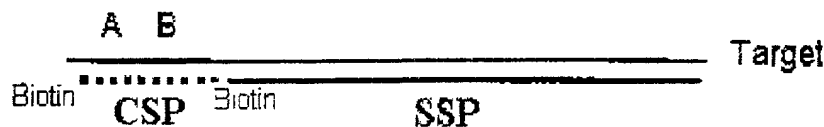


FIG. 1

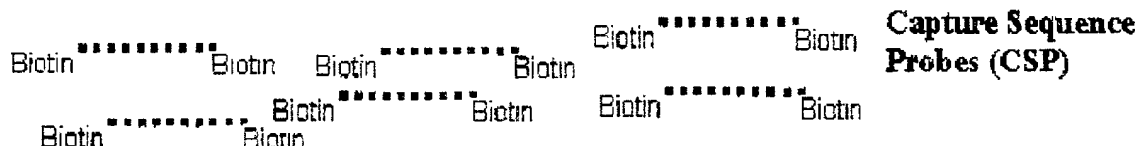
# Target Specific Hybrid Capture Utilizing Blocker Probe

(Simultaneous Hybridization Version)

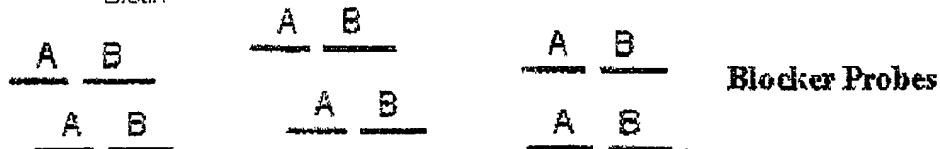
Hybridize Target  
with CSP and  
SSP



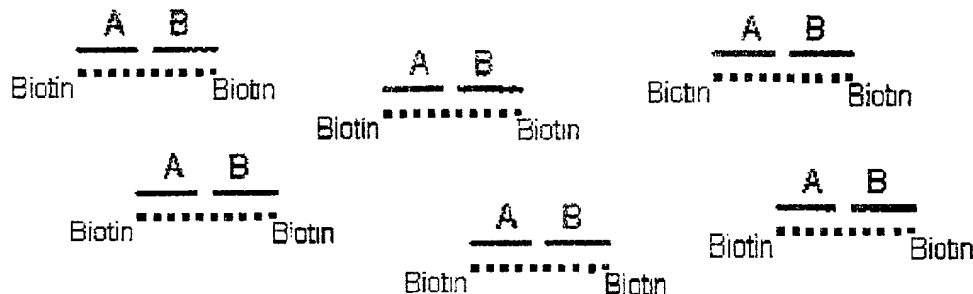
Excess CSP



Excess Blocker  
Probe



Cool Hyb in  
preparation for  
plate capture. At  
the reduced hyb.  
temperature the  
blocker probes  
hybridize to the  
free CSP,  
preventing them  
from hybridizing  
to cross-reactive  
targets in the  
sample.



Desired target  
molecule is not  
effected by the  
presence of the  
blocker probes

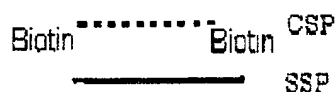
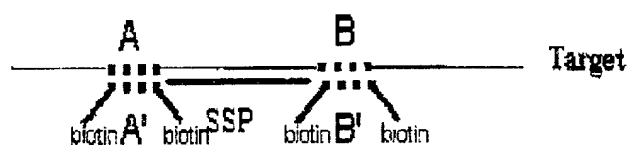


FIG. 2

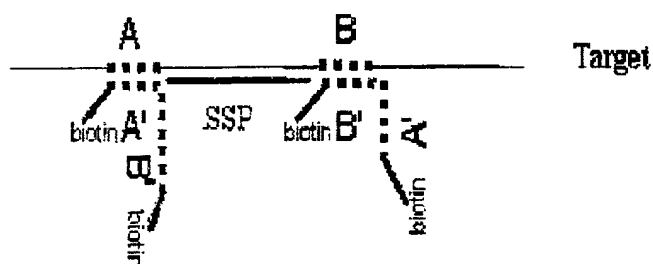


## TSHC with "Fused" Capture Sequence Probes (CSP)

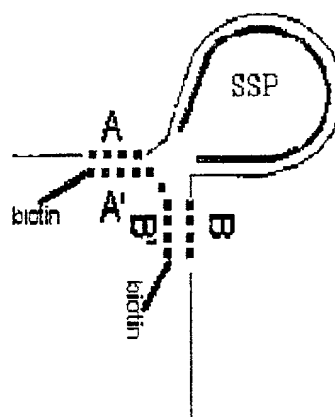
**Capture with two  
separate CSPs**



**Capture with two  
separate fusion  
CSPs**



**Capture with a  
single fusion CSP**



..... Unique Sequences

— Signal Sequence Probe

**FIG. 3**

# Analytical Sensitivity and Cross-reactivity of HSV-1 TSHC

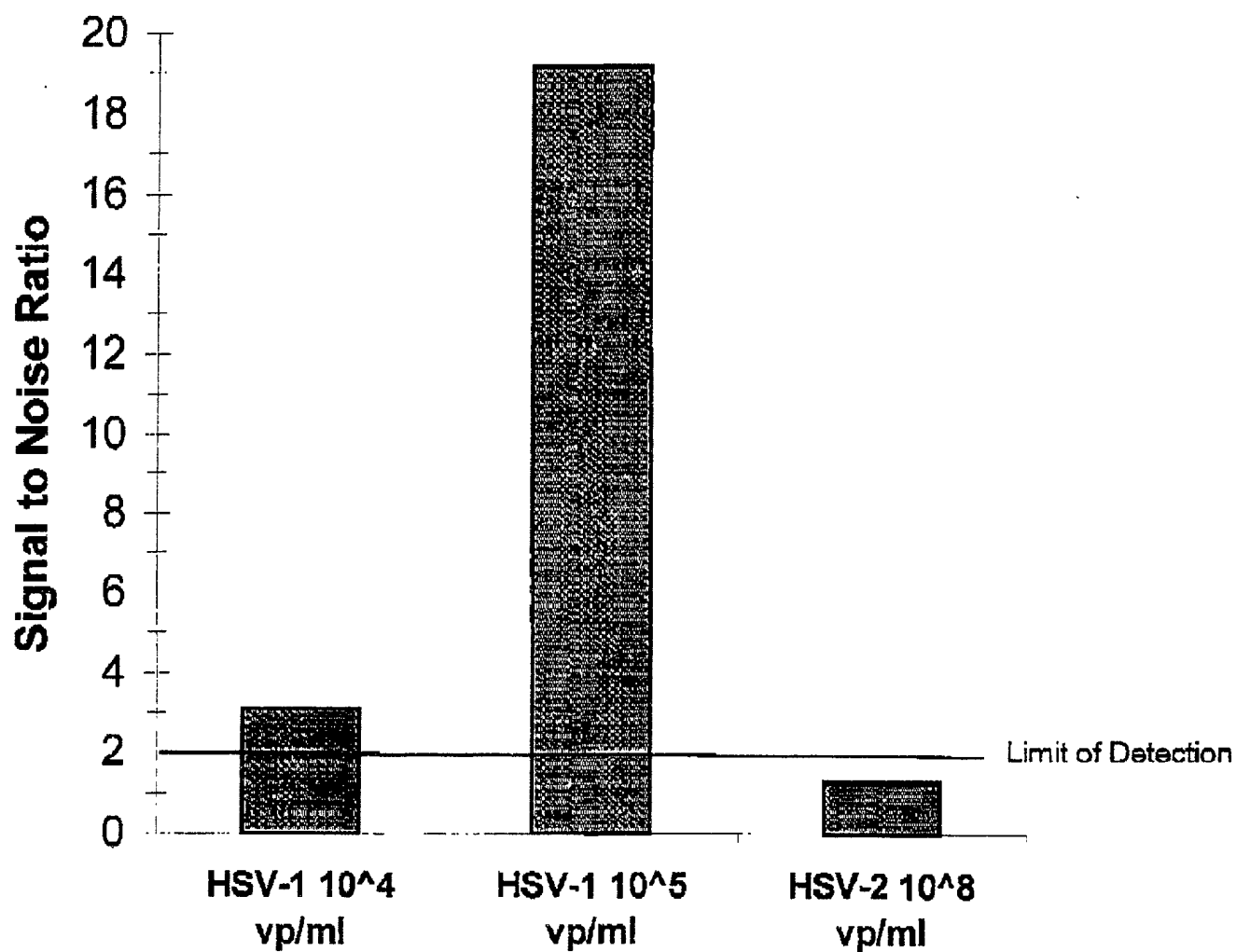


FIG. 4

## Analytical Sensitivity and Cross-reactivity of HSV-2 TSHC

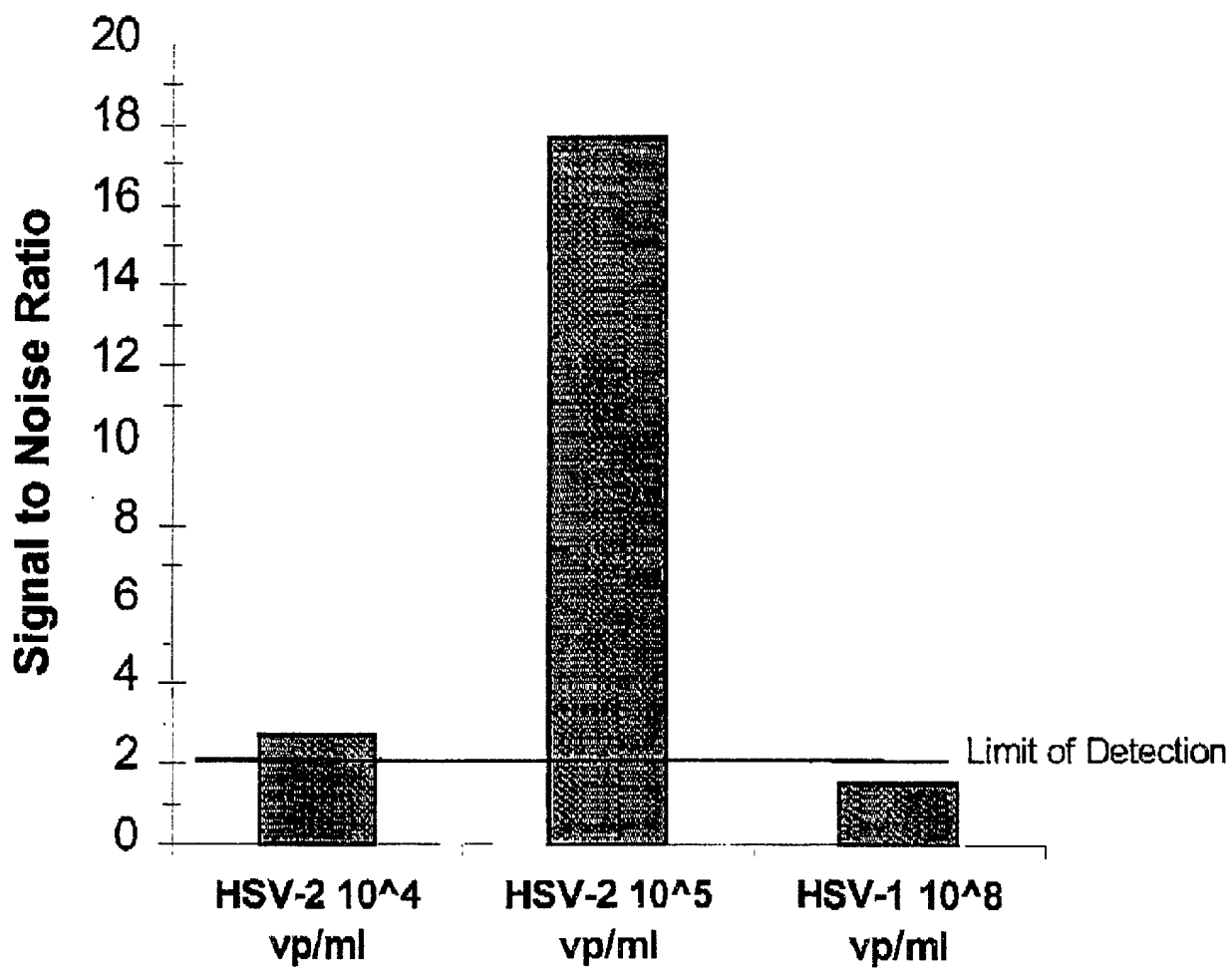


FIG. 5

# TSHC-Plus Version 1

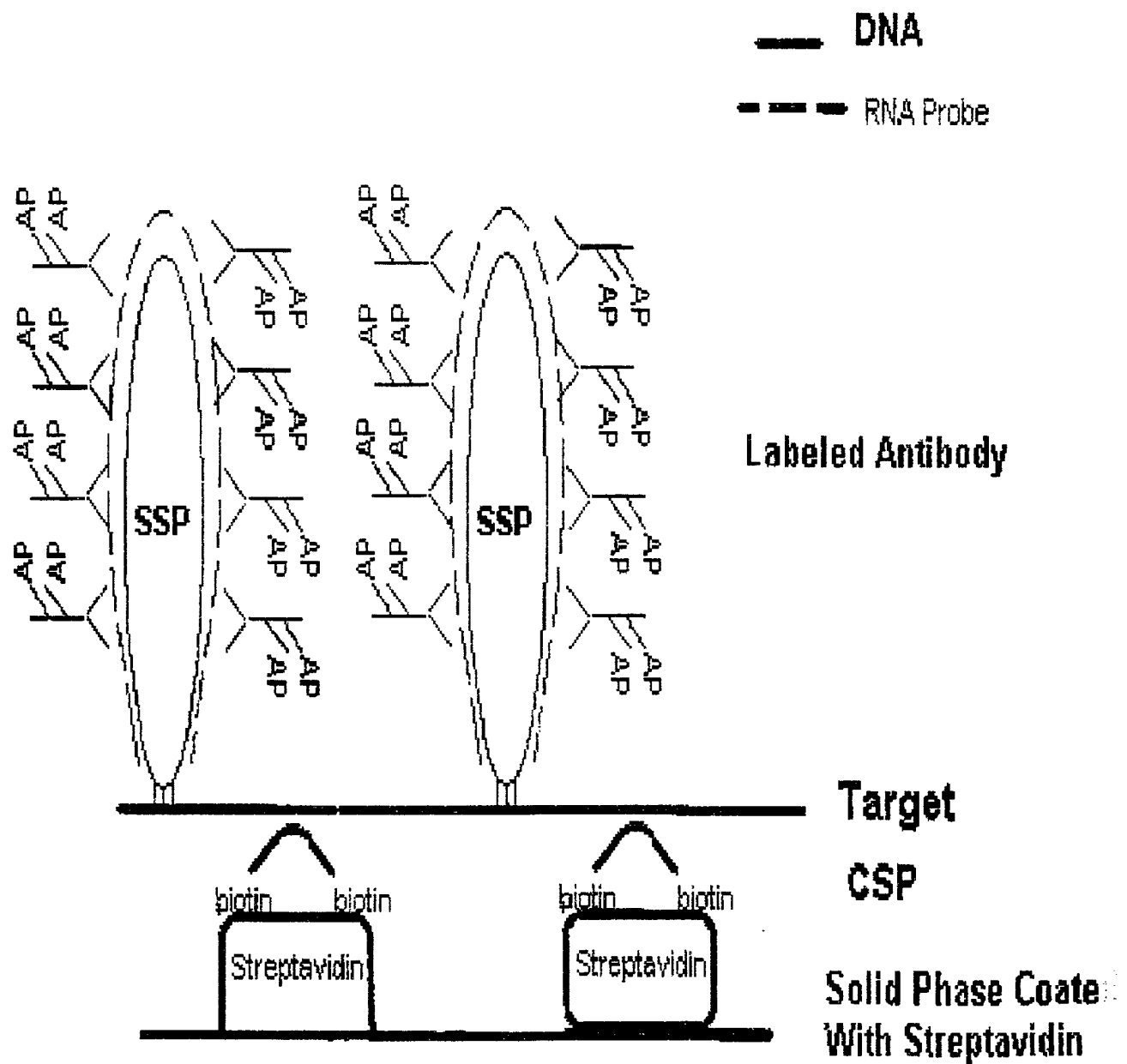


FIG. 6A

[illegible]



# TSHC-Plus Version 4

— · — Poly(dT)  
..... Poly(A)

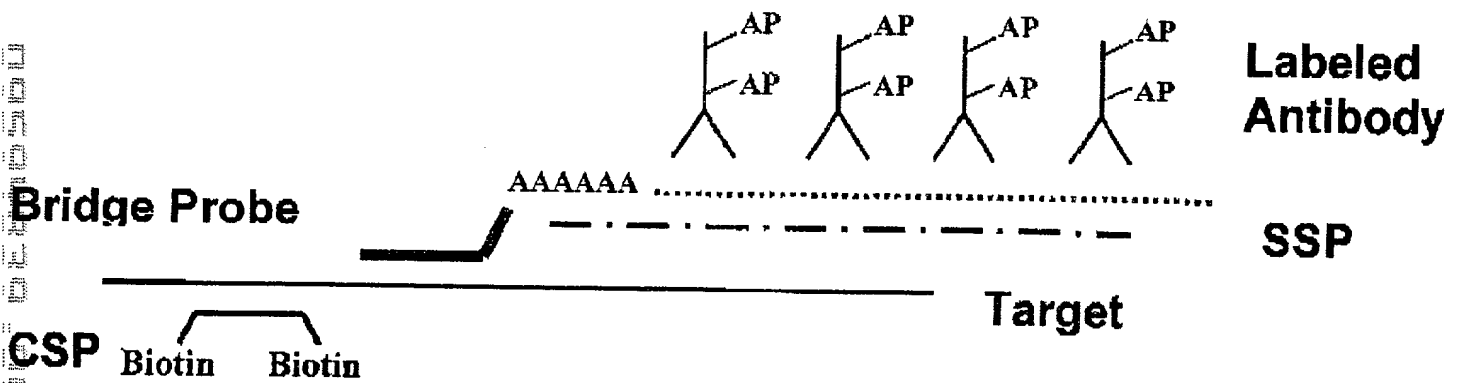


FIG. 6D

COMBINED DECLARATION AND POWER OF ATTORNEY FOR  
ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL  
DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION

As a below name inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

DETECTION OF NUCLEIC ACIDS BY TYPE-SPECIFIC HYBRID CAPTURE METHOD  
the specification of which

a. ☒ is attached hereto

b. ☐ was filed on \_\_\_\_\_ as application Serial No. \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

c. ☐ was described and claimed in International Application No. \_\_\_\_\_ filed on \_\_\_\_\_ and as amended on \_\_\_\_\_ (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby specify the following as the correspondence address to which all communications about this application are to be directed:

SEND CORRESPONDENCE TO: MORGAN & FINNEGAN, L.L.P  
345 Park Avenue  
New York, N.Y. 10154

DIRECT TELEPHONE CALLS TO: Dorothy R. Auth  
(212) 758-4800

☐ I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or under § 365(b) of any foreign application(s) for patent or inventor's certificate or under § 365(a) of any PCT international application(s) designating at least one country other than the U.S. listed below and also have identified below such foreign application(s) for patent or inventor's certificate or such PCT international application(s) filed by me on the same subject matter having a filing date within twelve (12) months before that of the application on which priority is claimed:

☐ The attached 35 U.S.C. § 119 claim for priority for the application(s) listed below forms a part of this declaration.



<u>Country/PCT</u>	<u>Application Number</u>	<u>Date of filing (day, month, yr)</u>	<u>Date of Issue (day, month, yr)</u>	<u>Priority Claimed</u>
--------------------	---------------------------	--	---------------------------------------	-------------------------

[ ] YES [ ] NO

[ ] YES [ ] NO

[ ] YES [ ] NO

[ ] I hereby claim the benefit under 35 U.S.C. § 119(e) of any U.S. provisional application(s) listed below.

Provisional Application No.

Date of Filing (day, month, yr)

**ADDITIONAL STATEMENTS FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART  
OR PCT INTERNATIONAL APPLICATION(S) (DESIGNATING THE U.S.)**

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or under § 365(c) of any PCT international application(s) designating the U.S. listed below.

<u>US/PCT Application Serial No.</u>	<u>Filing Date</u>	<u>Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)</u>
<u>US/PCT Application Serial No.</u>	<u>Filing Date</u>	<u>Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)</u>

[ ] In this continuation-in-part application, insofar as the subject matter of any of the claims of this application is not disclosed in the above listed prior United States or PCT international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

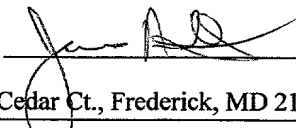
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or Imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and/or agents with full power of substitution and revocation, to prosecute this application, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith: John A. Diaz (Reg. No. 19,550), John C. Vassil (Reg. No. 19,098), Alfred P. Ewert (Reg. No. 19,887), David H. Pfeffer (Reg. No. 19,825), Harry C. Marcus (Reg. No. 22,390), Robert E. Paulson (Reg. No. 21,046), Stephen R. Smith (Reg. No. 22,615), Kurt E. Richter (Reg. No. 24,052), J. Robert Dailey (Reg. No. 27,434), Eugene Moroz (Reg. No. 25,237), John F. Sweeney (Reg. No. 27,471), Arnold I. Rady (Reg. No. 26,601), Christopher A.

Hughes (Reg. No. 26,914), William S. Feiler (Reg. No. 26,728), Joseph A. Calvaruso (Reg. No. 28,287), James W. Gould (Reg. No. 28,859), Richard C. Komson (Reg. No. 27,913), Israel Blum (Reg. No. 26,710), Bartholomew Verdirame (Reg. No. 28,483), Maria C.H. Lin (reg. No. 29,323), Joseph A. DeGirolamo (Reg. No. 28,595), Michael P. Dougherty (Reg. No. 32,730), Seth J. Atlas (Reg. No. 32,454), Andrew M. Riddles (Reg. No. 31,657), Bruce D. DeRenzi (Reg. No. 33,676), Michael M. Murray (Reg. No. 32,537), Mark J. Abate (Reg. No. 32,527), Alfred L. Haffner, Jr. (Reg. No. 18,919), Harold Haidt (Reg. No. 17,509), John T. Gallagher (Reg. No. 35,516), Steven F. Meyer (Reg. No. 35,613) and Kenneth H. Sonnenfeld (Reg. No. 33,285) of Morgan & Finnegan, L.L.P. whose address is: 345 Park Avenue, New York, New York, 10154; and Edward A. Pennington (Reg. No. 32,588), Michael S. Marcus (Reg. No. 31,727) and John E. Hoel (Reg. No. 26,279) of Morgan & Finnegan, L.L.P., whose address is 1775 Eye Street, Suite 400, Washington, D.C. 20006.

[ ] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from \_\_\_\_\_ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

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Inventor's signature\* 

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[ ] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from \_\_\_\_\_ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

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date

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Citizenship United States of America

Post Office Address same as above

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[ ] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from \_\_\_\_\_ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

Full name of sole or first inventor James Anthony

Inventor's signature\* \_\_\_\_\_

date

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Post Office Address same as above

Full name of second joint inventor, if any Attila Lorincz

Inventor's signature\* \_\_\_\_\_

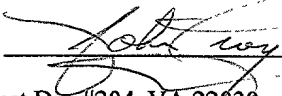
date

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Post Office Address same as above

Full name of third joint inventor, if any John Troy

Inventor's signature\* 

6/13/00

date

Residence 10725 West Dr., #204, VA 22030

Citizenship United States of America

Post Office Address same as above

Signed before me this 13th day of  
June, 2000  
Carol E. H. - Notary  
My Commission Expires 2003

ADDED PAGE TO COMBINED DECLARATION  
AND POWER OF ATTORNEY FOR  
SIGNATURE BY FOURTH AND SUBSEQUENT INVENTOR

Full name of fourth joint inventor, if any Yanlin Tang  
Inventor's signature\* *Yanlin Tang* 6/12/2000  
Residence 10621 Tuppence Court, Rockville, MD 20850 date  
Citizenship China  
Post Office Address same as above

- \* Before signing this declaration, each person signing must:
1. Review the declaration and verify the correctness of all information therein; and
  2. Review the specification and the claims, including any amendments made to the claims.
- After the declaration is signed, the specification and claims are not to be altered.

[ ] ATTACHED IS/ARE ADDED PAGE(S) TO COMBINED DECLARATION AND POWER OF ATTORNEY FORM FOR SIGNATURE BY FOURTH AND SUBSEQUENT INVENTORS

\* Before signing this declaration, each person signing must:

1. Review the declaration and verify the correctness of all information therein; and
2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

To the inventor(s):

The following are cited in or pertinent to the declaration attached to the accompanying application:

Title 37, Code of Federal Regulation, § 1.56

Duty to disclose information material to patentability.

(a) A patent by its very nature is affect with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

Title 35, U.S. Code § 101

Inventions patentable

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

## Title 35 U.S. Code § 102

### Conditions for patentability; novelty and loss of right to patent

A person shall be entitled to a patent unless –

- (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent,
- (b) the invention was patented or described in a printed publication in this or foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States, or
- (c) he has abandoned the invention, or
- (d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months before the filing of the application in the United States, or
- (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or
- (f) he did not himself invent the subject matter sought to be patented, or
- (g) before the applicant's invention thereof the invention was made in this country by another had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other ...

## Title 35, U.S. Code § 103

### Conditions for patentability; non-obvious subject matter

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

## Title 35, U.S. Code § 112 (in part)

### Specification

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms also enable any person skilled in the art to which it pertains, or with which it is mostly nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Title 35, U.S. Code § 119

Benefit of earlier filing date in foreign country; right of priority

An application for patent for an invention filed in this country by any person who has, or whose legal representatives or assigns have, previously regularly filed an application for a patent for the same invention in a foreign country which affords similar privileges in the case of applications filed in the United States or to citizens of the United States, shall have the same effect as the same application would have if filed in this country on the date on which the application for patent for the same invention was first filed in such foreign country, if the application in this country is filed within twelve months from the earliest date on which such foreign application was filed; but no patent shall be granted on any application for patent for an invention which had been patented or described in a printed publication in any country more than one year before the date of the actual filing of the application in this country, or which had been in public use or on sale in this country more than one year prior to such filing.

Title 35, U.S. Code § 120

Benefit or earlier filing date in the United States

An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, or as provided by section 363 of this title, which is filed by an inventor or inventors named in the previously filed application shall have the same effect, as to such invention, as though filed on the date of the prior application, if filed before the patenting or abandonment of or termination of proceedings on the first application or an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application.

Please read carefully before signing the Declaration attached to the accompanying Application.

If you have any questions, please contact Morgan & Finnegan, L.L.P.

FORM:COMB-DEC.NY  
Rev. 5/21/98



SEQUENCE LISTING

<110> Anthony, James  
Lorincz, Attila  
Troy, John  
Tang, Yanlin

<120> DETECTION OF NUCLEIC ACIDS BY TARGET-SPECIFIC HYBRID  
CAPTURE METHOD

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<213> Artificial Sequence

<220>

<223> SG-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 62

cgggacggcg agcggaaaagt caacgt

26

<210> 63

<211> 9

<212> DNA

<213> Artificial Sequence

<220>

<223> HX-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 63

ggcgcgggc

9

<210> 64

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> HX-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 64

gaaagtagtg ctggc

15

<210> 65

<211> 11

<212> DNA

<213> Artificial Sequence

<220>

<223> GP-7

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 65

tgctggcggc g

11

<210> 66

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> AZ-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 66

acacctccca acg

13

<210> 67

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> AZ-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 67

ctccaggatg acg

13

<210> 68

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> GR-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 68

tcggttttgg tc

12

<210> 69

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> GR-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 69

acacagaacc tca

13

<210> 70

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> GP-8

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 70

cacacacctc cca

13

<210> 71

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> BR-10

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 71



cgaccccccta gata

14

<210> 72

<211> 11

<212> DNA

<213> Artificial Sequence

<220>

<223> BR-11

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 72

ccacgcgctcg g

11

<210> 73

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> HX-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 73

acgttgactt tccgc

15

<210> 74

<211> 10

<212> DNA

<213> Artificial Sequence

<220>

<223> BR-15

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 74

cgccgtcccg

10

<210> 75

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> ZL-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 75

gtacagatgg taccgggggtt gtagaagtat ctg

33

<210> 76

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> ZL-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 76

ctgcaacaag acatacatcg accggtccac c

31

<210> 77

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> DP-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 77

gaagtaggtg aggctgcatg tgaagtggta g

31

<210> 78

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> DP-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 78

cagctctgtg cataactgtg gtaactttct ggg

33

<210> 79

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> SH-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 79

gaggtcttct ccaacatgct atgcaacgtc ctg

33

<210> 80

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> SH-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 80

gtgtaggtgc atgctctata ggtacatcag gcc

33

<210> 81

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> VS-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 81

caatgccgag cttagtcat gcaatttcg agg

33

<210> 82

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> VS-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 82

gaagtagtag ttgcagacgc ccctaaaggt tgc

33

<210> 83

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> AH-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 83

gaacgcgatg gtacaggcac tgcagggtcc

30

<210> 84

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> AG-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 84

gaacgcgatg gtacaggcac tgca

24

<210> 85

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> AL-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 85

acgcccaccc aatggaatgt accc

24

<210> 86

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> PA-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 86

tctgcgtcgt tggagtcgtt cctgctcgtc tc

32

<210> 87

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-1AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 87

ttattattac tacatacatt gccgccatgt tcgcca

36

<210> 88

<211> 46

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-2AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 88

ttattattat gttgccctct gtgcccccggt tgtctatagc ctccgt

46

<210> 89

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-3AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 89

ttattattag gagcagtgcc caaaagatta aagtttgc

38

<210> 90

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-4AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 90

ttattattac acggtgctgg aatacgggtga ggggggtg

37

<210> 91

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-5AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 91

ttattattaa cggccaccca atggaatgta ccc

33

<210> 92

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-6AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 92

ttattattaa tagtattgtg gtgtgtttct cacat

35

<210> 93

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-7AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 93

ttattattag ttggagtcgt tcctgtcgtg

30

<210> 94

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> 18-8AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 94

ttattattac ggaatttcat tttggggctc t

31

<210> 95

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> PE-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 95



gctcgaaggt cgtctgctga gctttctact act 33

<210> 96  
 <211> 32  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> PZ-2

<220>  
 <223> Description of Artificial Sequence:nucleic acid  
 probe

<400> 96  
 gcgccatcct gtaatgcact tttccacaaa gc 32

<210> 97  
 <211> 31  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> PZ-5

<220>  
 <223> Description of Artificial Sequence:nucleic acid  
 probe

<400> 97  
 tagtgctagg tgtagtggac gcaggaggtg g 31

<210> 98  
 <211> 32  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> CS-1

<220>  
 <223> Description of Artificial Sequence:nucleic acid  
 probe

<400> 98

ggtcacaaca tgtattacac tgccctcggt ac

32

<210> 99

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> CS-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 99

cctacgtctg cgaagtcttt cttgccgtgc c

31

<210> 100

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> PF-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 100

ctgcattgtc actactatcc ccaccactac ttg

34

<210> 101

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> PF-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 101

ccacaaggca cattcataca tacacgcacg ca

32

<210> 102

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> PA-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 102

gttctaagggt cctctgccga gctctctact gta

33

<210> 103

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> 45-5AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 103

ttattattat gcggttttgg gggtcgacgt ggaggc

36

<210> 104

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> 45-6AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 104

ttattattaa gacctgcccc ctaagggtac atagcc

36

<210> 105

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> 45-8AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 105

ttattattac agcattgcag cctttttggt acttgcttgt aatagctcc

49

<210> 106

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> 45-9AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 106

ttattattaa toctgtaatg cacttttcca caaa

34

<210> 107

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> 45-10AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 107

ttattattag cctggtcaca acatgtatta c

31

<210> 108

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> 45-11AB

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 108

ttattattac aggatctaatt tcattctgag gtt

33

<210> 109

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> ON-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 109

tgcggttttg ggggtcgacg tggaggc

27

<210> 110

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> PV-FD-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 110

gcctccacgt cgac

14

<210> 111

<211> 11

<212> DNA

<213> Artificial Sequence

<220>

<223> PV-FD-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 111

ccccaaaacc g

11

<210> 112

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PV-FD-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 112

ggtacattcc attggg

16

<210> 113

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PV-FD-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 113

tgggcgtaa taataa

16

<210> 114

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> AH-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 114

accatcgcggt tc

12

<210> 115

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> AH-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 115

ggaccctgca gtgc

14

<210> 116

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> AH-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 116

ctgtaccatc gcgtt

15

<210> 117

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> AH-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 117

tgcagtgctt gt

12

<210> 118

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> PZ-1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 118

ccacctcctg cgt

13

<210> 119

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> PZ-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 119



attacaggat ggcgc

15

<210> 120

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PZ-4

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 120

gctttctgga aaagtg

16

<210> 121

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> PZ-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 121

ccactacacc tagcacta

18

<210> 122

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> ZL-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 122

cagatacttc tacaacc

17

<210> 123

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> ZL-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 123

ccggtaccat ctgtac

16

<210> 124

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> ZL-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 124

ggtggaccgg tcg

13

<210> 125

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> ZL-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 125

atgtatgtct tgttgcag

18

<210> 126

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> DP-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 126

ctaccacttc acatgc

16

<210> 127

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> DP-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 127

agcctcacct acttc

15

<210> 128

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> DP-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 128

cccagaaagt taccac

16

<210> 129

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> DP-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 129

agttatgcac agagct

16

<210> 130

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> SH-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 130

caggacgttg catagc

16

<210> 131

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> SH-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 131

atgttggaga agacctc

17

<210> 132

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> SH-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 132

ggcctgatgt acctata

17

<210> 133

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> SH-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 133

gagcatgcac ctacac

16

<210> 134

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> VS-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 134

ctcggaaatt gcatg

15

<210> 135

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> VS-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 135

aactaagctc ggcatt

16

<210> 136

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> VS-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 136

gcaaccttta gggg

14

<210> 137

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> VS-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 137

cgtctgcaac tactacttc

19

<210> 138

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> CS-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 138

gtaccgaggg cagt

14

<210> 139

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> CS-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 139

gtaatacatg ttgtgacc

18

<210> 140

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> CS-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 140

ggcacggcaa gaaa

14

<210> 141

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> CS-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 141

gacttcgcag acgtagg

17

<210> 142

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> PF-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 142

caaagtagtg gtggg

15

<210> 143

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> PF-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 143



gatagtagtg acaatgcag

19

<210> 144

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PF-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 144

tgcgtgcgtg tatgta

16

<210> 145

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PF-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 145

tgaatgtgcc ttgtgg

16

<210> 146

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> PE-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 146

agtagtagaa agctcagc

18

<210> 147

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> PE-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 147

agacgacctt cgagc

15

<210> 148

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> PA-2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 148

tacagtagag agctcgg

17

<210> 149

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PA-3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 149

cagaggacct tagaac

16

<210> 150

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PA-5

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 150

gagcacgaca ggaacg

16

<210> 151

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> PA-6

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 151

actccaacga cgcaga

16

<210> 152

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> HBV C1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 152

gctggatgtg tctgcggcgt tttatcat

28

<210> 153

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> HBV C2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 153

actgttcaag cctccaagct ggcgctt

27

<210> 154

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> HBV C3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 154

atgataaaaac gccgcagaca catccagcga ta

32

<210> 155

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> B1

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 155

atgataaaaac gccg

14

<210> 156

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> B2

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 156

cagacacatc cagc

14

<210> 157

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> B3

<220>

<223> Description of Artificial Sequence:nucleic acid  
probe

<400> 157

aaggcacagc ttg

13

<210> 158

<211> 14

<212> DNA

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